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Genetic Factors in Parabiosis [1, 2]

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SUMMARY

Parabiotic compatibility is primarily dependent on identical alleles at the *H-2* locus. "Bilateral" difference at the *H-2* locus—*each* partner having an *H-2* allele not carried by the other—results in separation of partners during or after the 2d week of union. Separation is preceded by cessation of dye transmission (Evans Blue and Hypaque) from the 6th day on. Persistence of union beyond the usual time of separation, "false union," is merely passive and not accompanied by dye transmission. "Unilateral" difference at the *H-2* locus, one partner having an *H-2* allele not carried by the other, as in many "pure" F_1 hybrid unions, usually results in "parabiotic intoxication" of the partner carrying the foreign allele. Death commonly occurs during the latter half of the 2d week or the first half of the 3d week. Dye transmission ceases early during the 2d week. Dissimilarity in sex, at the *H-1* or the *H-3* locus, does not result in separation or intoxication. In all groups compatible at the *H-2* locus Hypaque transmission has been observed for at least 2 months after union. The subject of parabiotic intoxication is discussed.—J. Nat. Cancer Inst. 23: 1193–1213, 1959.

MOST REPORTS on parabiosis are concerned with the extent to which one animal participates in the biological processes of the partner (*1*). Little has been reported on the nature of the parabiotic union itself, though it seems likely that the sequelae of the union are largely determined by the selection of partners and especially by their genetic relationship. The influence of the genetic relationship on parabiotic union of mice has not been systematically investigated heretofore, and is the subject of this report.

Parabiotic union represents a special form of host-graft relationship. It offers certain advantages not shared by other experimental techniques applied in the study of host-graft relationships. It permits differentiation

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of direct adverse effects of a host against a graft from phenomena merely due to vascular obstruction. Even the most vigorous reaction of a host against a graft, involving complete thrombotic obliteration of the blood supply to the graft bed, does not deprive the graft of its nourishment. Also, the presence and extent of graft-*versus*-host reactions frequently remain unknown in ordinary grafting procedures. This problem does not exist in parabiosis: A graft-*versus*-host reaction must always be taken into account and is equal in importance to the host-*versus*-graft reaction.

It has been reported that parabiotic union induces a state of at least relative tolerance (*2*) affecting both the union itself and any secondary procedures that may be undertaken after union has been accomplished: Genetically heterogeneous partners are stated to have remained in union long after an expected adverse immune reaction should have taken place and to have permanently accepted skin transplants, either during union or after an episode of union, which otherwise would have been rejected. The belief that parabiotic union produces tolerance of skin grafts is in part justified, as we shall report elsewhere. The claim that parabiotic union may persist in the presence of genetic dissimilarity of partners also is in part justified. However, we believe that this claim has, at times, been made because the criteria of union have been too loose, and the methods applied in evaluating the state of union have lacked sufficient sensitivity.

MATERIALS AND METHODS

Mice of the C57BL/6, A, ST, C3H, BALB/c, and CBA strains, and their F_1 hybrids, were used. The original breeding stock, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, has been maintained in our laboratory for several years, by brother × sister matings. Mice of isogenic resistant lines were obtained from Dr. George Snell who provided mice of the C57BL/10 and C57BL/10.D2 lines, differing at the *H-2* locus, mice of the C3H and C3H/K lines, differing at the *H-1* locus, and mice of the C57BL/10 and C57BL/10.LP lines differing at the *H-3* locus. At the time of parabiosis all mice were at least 60 days old. Unless specified otherwise, partners were of the same sex, female pairs predominating, to minimize the effect of trauma on the junctional zone caused by the more vigorous males.

Parabiosis was performed by a modified Bunster and Meyer method while the mice were under chloral hydrate anesthesia, with 20,000 units of penicillin being administered at the same time. After clipping and depilating, skin and panniculus were incised from knee to elbow. The subpannicular space was dissected bluntly for about one centimeter in all directions. This required caution in the axillary tissue to prevent bleeding. The peritoneal cavities were not entered. A #4–0 chromic catgut ligature was placed around the knees and elbows, and the skin and panniculus were joined by continuous #4–0 chromic catgut suture. This technique

ensured ample contact between the apposed subpannicular surfaces (fig. 1), and resulted in the formation of a capillary cavity between partners, referred to later as "interpartner cavity." Most of the pairs were permitted to survive until separation, death, or, in apparent permanent union, for at least 8 weeks. Other pairs were killed for histologic studies.

For histologic studies, sections of the junctional zone were prepared by placing it over a small wooden block and excising a generous square of the adjacent tissues. When indicated, a suture was placed through the tissues of one partner as a marker. The tissues were fixed in 10 percent formalin; sections were stained with hematoxylin and eosin.

Blood for erythrocyte counts was obtained from a tail vein. Counts were done with an Evelyn photometer after a curve had been established on the basis of multiple chamber counts.

Transmission studies were performed with chicken erythrocytes, Trypan Blue (0.01 to 1.0%, in Ringer's solution), Evans Blue (T-1824, 0.5%, in Ringer's solution), and a radiopaque dye (Hypaque, Winthrop Laboratories). Chicken erythrocytes disappeared too rapidly from the peripheral blood of the injected animal to be readily detectable in the partner. Trypan Blue was more toxic than Evans Blue and offered no advantages over the latter.

Transmission of intraperitoneally injected Evans Blue (0.45 ml.) proved a sensitive method to determine the degree of union. Transmission was either absent, limited to the adjacent tissues of the uninjected partner ("local transmission"), or systemic. In systemic transmission there was discoloration of the partner's vulva, mammillae, conjunctivas, retinas, footpads, nose, tail, and skin, listed in their approximate order of appearance. The endpoint was reasonably sharp, if read 24 hours after injection. After 48 hours there was occasionally some systemic distribution of dye within an uninjected partner that originally had shown only local transmission. Evans Blue did not affect survival or time of separation in incompatible partners.

Transmission of Hypaque, studied radiographically, was at least as sensitive, provided a sharper endpoint than Evans Blue, and could be repeated daily. If the dye was injected intravenously into a single mouse, the kidney shadows became visible in about 20 minutes, and the bladder shadow soon thereafter. All visible dye had disappeared within 24 hours after injection. After subcutaneous injection of 0.5 ml.—the injection site being remote from the peritoneal cavity to prevent interference with the radiological appearance of the urinary tract—the kidney and bladder shadows appeared somewhat later and persisted longer. If injected into a compatible pair, the dye was easily seen in the uninjected partner almost as soon as in the injected one, though generally somewhat weaker (fig. 5). Of the two injection routes, the subcutaneous was superior to the intravenous, presumably because of the protracted absorption from the injection site. Frequently transmission occurred after a subcutaneous injection even though transmission failed to take place after similar pairs received intravenous injection. An occasional false negative was en-

countered due to early emptying of the bladder. In repeated injections, films were taken prior to injection to ascertain the disappearance of previously injected dye.

The sensitivity of the Hypaque method was attested to by a pure-hybrid pair whose line of junction had torn extensively on the 7th day after union. The pair was joined only by the forelegs and a small amount of subpannicular tissue in back of the forelegs, but Hypaque nevertheless was readily transmitted.

OBSERVATIONS

Partners of Same Strain and Sex

Partners of the same strain and sex (table 1) usually developed persistent, "true," union. The line of junction usually healed well, with little crusting or seeping. Occasional areas of dehiscence healed by contraction. Hair growth in the depilated areas became visible within 3 weeks and at times was sufficiently luxurious to interfere with the easy identification of the junctional zone. Palpation of the junctional zone, after 1 month, failed to reveal features of note; the dorsal and ventral layers were freely movable against each other.

A mild to moderate postoperative anemia was commonly noted, but erythrocyte levels soon reverted to normal levels. Differences in skin color at the line of junction were seen occasionally, with one partner appearing congested and the other pale, but this color contrast was neither severe nor persistent. In partners killed during the first 10 days, mild to moderate splenomegaly was observed, with patches of myelopoiesis in the enlarged spleen. Enlargement of the lymph nodes was usually confined to the nodes adjacent to the junctional zone.

Pairs killed during the 1st week after union showed a severe inflammatory response, chiefly in the abutting subpannicular spaces, with accumulation of fibrinous exudate and inflammatory cells around and within the interpartner cavity, and with early necrosis of the skin edges raised by the surgical suture. The severed pannicular edges showed necrosis, with focal regeneration and proliferation of fibroblasts in the perimysial structures. As the acute inflammation subsided, regenerative hyperplasia of the epidermis and thickening of the dermal collagen occurred. The interpartner cavity was gradually obliterated and replaced by a narrow fibrous band covered dorsally and ventrally by the well-healed pannicular layers. The pannicular layers between partners usually fused incompletely. Slight pannicular fibrosis extending toward the epidermis indicated the zone of junction (fig. 3).

Evans Blue injected immediately on completion of the parabiotic procedure was transmitted to the partner, but only to those tissues adjacent to the line of junction. In one instance, one partner was killed immediately after parabiosis, but left in union with the survivor. The survivor

TABLE 1.—Partners of same strain and sex

Strain	Number of pairs	Number of pairs killed for histology	Number of pairs used for dye transmission	Number of pairs observed 30 days or more	Day of separation	Day of death—*both* dead	Day of death—*one* dead	Number of pairs with "early disease"*
BALB/c	19	7	11	3		8, 8, 11, 19, 26, 46	16	2
ST	11	5	9	8				
A	10	8	8	6				
(ST × C57BL)F$_1$†	1			1				
Total	41	20	28	18		6 pairs	1 pair	2

*Six Observations.
†Or reciprocals.

was injected with Evans Blue and killed 3 hours later. The pair was refrigerated for several hours during which time local transmission occurred. This was very likely the result of passive diffusion of the dye and not indicative of an active exchange of body constituents. Dye injected on the 1st or 2d postoperative day was also transmitted locally. Dye injected on the 3d day and thereafter was transmitted systemically to the partner, at first faintly, but more noticeably on successive days. The transmission of Hypaque followed a similar pattern and was markedly in evidence from the 3d day on.

Partners Differing Bilaterally at the *H-2* Locus

Whenever partners differed bilaterally at the *H-2* locus (table 2), dehiscence of the line of junction occurred. Since temporary dehiscence was also seen in some compatible pairs, its onset was not interpreted as a sign of incompatibility. Dehiscence increased gradually though seemingly interrupted by episodes of crusting and contraction. Not infrequently a dehiscence at the dorsal line of junction faced another at the ventral aspect, so that the bottom of the cage became visible between the partners. Final separation most frequently took place between the 13th and 15th days. Occasionally partners remained united for several days by only a narrow pedicle, consisting of dense fibrous tissue and enclosing occasionally, but not always, fragments of suture material. At times the partners appeared to remain in union for prolonged periods of time, up to 68 days. In such cases persistence of union was merely passive and associated with the presence of thick crusts and dense fibrous tissue gluing the animals together and preventing the exchange of body constituents. This state has been aptly described by Andresen in parabiotically joined rabbits (*3*). We have designated it as one of "false union" (*see* transmission studies later).

An occasional difference in skin color at the line of junction was observed, but its distribution did not appear to be influenced by the strain of the partners. The color contrast during such periods, as in isologous pairs, was of short duration, not severe, and not consistently associated with changes in the erythrocyte level.

When killed after separation or during the 2d or 3d week of union, the spleens of both partners were enlarged and showed widespread myeloid activity. Curiously in pairs of BALB/c and ST mice, our most frequent combination, the BALB/c spleens were larger than those of their partners during the first 10 days of union, while the comparative size was reversed thereafter.

Histologically, the events during the 1st week did not differ appreciably from those seen in compatible partners. Toward the end of the 1st week there was increasing subpannicular edema. Lymphocytes and large mononuclear cells seemingly fanned out from the frayed edges of the severed panniculus and filled the space between the epidermal junction and the interpartner cavity. From the 12th day on, a clear zone was

TABLE 2.—Partners differing at *H-2* locus

Strain	Number of pairs	Number of pairs killed for histology	Number of pairs used for dye transmission	Number of pairs observed 30 days or more	Day of separation	Day of death—*both* dead	Day of death—*one* dead	Number of pairs with "cancer disease"*
C57BL–ST	11			2	11, 15, 17, 18, 26, 28, 28, 28, 29, 36, 68			
BALB/c–ST	42	13	19	5	6, 14, 15, 16, 17, 22, 23, 23, 30, 30, 37, 39	5, 6, 6, 7	2, 4, 5, 5, 5, 6, 6, 6, 8, 8, 11, 14	12
C57BL–BALB/c (BALB/c × A)F_1–(CBA × C57BL)F_1†	1						12	
A–BALB/c	11		6		7, 24		7, 7, 7, 8, 9	3
A–(ST × C57BL)F_1†	4				14, 14, 15, 17			
A × (A × C57BL)F_1–C57BL × (C57BL × A)F_1†	1						15	
ST–A × (A × C57BL)F_1†	2			1			6, 30	1
C57BL/10–C57BL/10. D2	1						23	
	8		4	4	21, 23, 24, 25, 33, 39, 40, 42			
Totals	81	13	29	12	33 pairs	5 pairs	22 pairs	16

*See Observations.
†Or reciprocals.

noted between the partners, consisting of a partly homogeneous, partly granular precipitate in which a few polymorphonuclear leukocytes and macrophages were scattered about. By the end of the 2d week the leukocytes had increased. Many showed nuclear oversegmentation, pyknosis, and other degenerative signs; they formed a septum transecting the clear field between partners and connecting the lower surface of the epidermis with the cavity below (fig. 2). If union persisted beyond the 2d week, the septum shortened and was partially replaced by dense fibrous tissue. Aside from congestion, no vascular changes were observed.

Systemic transmission of Evans Blue and Hypaque began on the 3d day of union, as with compatible partners. From the 6th day on, transmission of Evans Blue again became limited to the tissues adjacent to the junctional site, similar to that seen during the first 2 days of union. If union persisted beyond the 2d week (false union), transmission ceased altogether at the junctional site (fig. 4). Transmission of Hypaque also ceased on the 6th day (fig. 6).

Partners Differing Unilaterally at the *H-2* Locus

Union of a mouse of one strain (pure) with its F_1 hybrid derived from a cross with a strain differing at the *H-2* allele (table 3) resulted in parabiotic intoxication of the hybrid partner, with death usually occurring between the 12th and 19th days of union. Rarely did the hybrid survive beyond the end of the 3d week. This course of events occurred whenever a difference at the *H-2* locus was involved, irrespective of the strains concerned. The picture of intoxication was similar to that described by other authors (*1*). The hybrid showed hunching and ruffling of its fur about the 7th day, with blanching of its skin as compared to the pure partner. This was accompanied by a precipitous drop in erythrocyte count, while that of the partner showed a moderate increase (text-fig. 1).

At death the hybrid had congested viscera, a spleen of slightly increased size (mean $\pm$ S.D.: 205 $\pm$ 84 mg.), and grossly normal lymphoid tissue. The pure partner, when killed at the time of death of the hybrid, showed a moderately to markedly enlarged spleen (mean $\pm$ S.D.: 348 $\pm$ 151 mg.). This increase in weight was associated with marked proliferation of myeloid cells. The remaining viscera, particularly the lymphoid tissue, kidneys, adrenals, lungs, liver, and heart, showed no microscopic features of note.

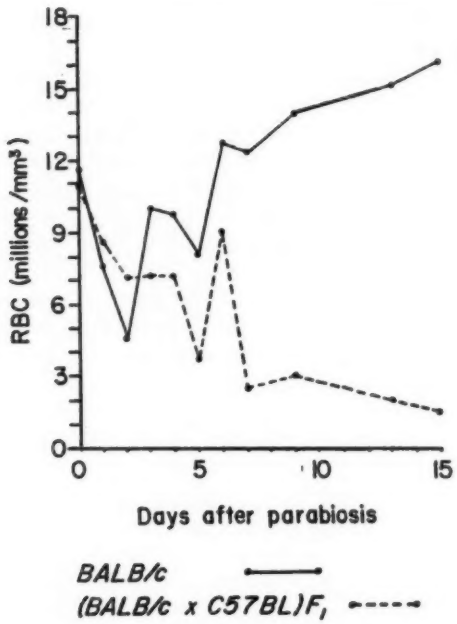
Systemic transmission of Evans Blue and Hypaque, as in compatible and incompatible pairs, began around the 3d day and ceased around the 8th or 9th day, about 2 days later than in bilaterally incompatible partners. Transmission from pure to hybrid ceased at the same time as transmission in the opposite direction.

The histologic appearance of the junctional zone during the first 10 days did not differ significantly from that of bilaterally incompatible partners. However, the clear zone and junctional septum seen in incompatible partners during the latter half of the 2d week did not develop; instead there

TABLE 3.—Pure F_1 hybrid partners*

Strain	Number of pairs	Number of pairs killed for histology	Number of pairs used for dye transmission	Number of pairs observed 30 days or more	Day of separation	Day of death—*both* dead	Day of death—pure died first	Day of death—F_1 died first	Number of pairs with "early disease"†
ST–(ST × C57BL)F_1	6							12, 12, 13, 15, 15, 17	
A–(A × C3H)F_1‡	8			1		5, 14, 25	21	12, 21, 25, 38	1
A–(BALB/c × A)F_1‡	1				7				
BALB/c–(BALB/c × A)F_1‡	1				7				
C57BL–(C57BL × ST)F_1‡	6							14, 15, 16, 16, 16, 19	
C57BL–(BALB/c × C57BL)F_1‡	4							11, 12, 15, 16	
BALB/c–(BALB/c × C57BL)F_1‡	61	2	11		11	6, 6, 6, 6, 7, 7, 13, 13	5, 5, 6, 6, 6, 6, 7, 7, 7, 8, 8, 11, 11, 12, 13, 13, 15, 25, 27	4, 4, 8, 9, 9, 10, 10, 11, 12, 12, 13, 13, 13, 13, 14, 14, 14, 15, 15, 16, 16, 17, 18, 19, 27	18
ST–(ST × A)F_1‡	3							12, 15	
C57BL–(C57BL × A)F_1‡	11	4	8			10		11, 11, 13, 13, 17, 25	
Totals	101	6	19	1	3	12	21	55	19

*Other parental strain differing at *H-8* locus.
†See Observations.
‡Or reciprocals.



TEXT-FIGURE 1.—Mean erythrocyte levels of 4 pure-hybrid pairs in parabiosis.

was persistence and intensification of the inflammatory response, with increased infiltration and proliferation of lymphocytes, fibroblasts, and large mononuclear cells.

Partners Differing Only in Sex, at *H-1* or at *H-3* Locus

When partners differed only at the *H–1* or at the *H–3* locus, or differed in sex and were of a strain in which females are known to reject male skin grafts (table 4), the external course resembled that seen in partners identical in strain and sex: In most instances the line of junction healed well, union appeared permanent, and transmission of Hypaque persisted beyond the time at which it is known to cease between partners differing at the *H–2* locus. There was, however, some variation in dye transmission during later stages. In most partners differing only at the *H–3* locus, the ability to transmit Hypaque seemed to persist permanently and was observed as late as 161 days after junction in a CBA–ST pair. In partners differing only at the *H–1* locus, the results were less clear-cut. There were episodes during which transmission appeared faint or absent, as early as the 12th day, only to reappear, in the same pair, at a later date. However, transmission has persisted in pairs tested as late as the 298th day. In one pair differing only in sex and of the appropriate genetic background (*see* preceding) it had faded out by the 26th day. In another

TABLE 4.—Partners differing only in sex, at *H-1* or *H-3* locus

Strain	Number of pairs	Number of pairs killed for histology	Number of pairs used for dye transmission	Number of pairs observed 30 days or more	Day of separation	Day of death—*both* dead	Day of death—*one* dead	Number of pairs with "early disease"*
Sex								
(C57BL–A)F_1†	8			5	5	27, 52	63, 37, 51, 17	
C57BL	8	1	2	7		31, 48, 95		
(C3H × C57BL)F_1†	2			2				
(ST × C57BL)F_1†	3		2	3				
(BALB/c × C57BL)F_1†	4		1	4		40		
(CBA × C57BL)F_1†	1	1	1	1				
H-1								
C3H.K–C3H	8		5	6		150, 166	4, 7	2
H-3								
C57BL/10–C57BL/10.LP								
CBA–C3H‡	3	1	1	1		12, 20		
CBA–ST‡	4		2	1			9, 27, 96	
Totals	41	3	14	30	1	10	9	2

*See Observations.

†Or reciprocals.

‡Probably differing only at *H-3* locus (personal communication, G. Snell).

pair it had ceased by the end of the 6th week. In others, dye was still transmitted freely at the end of 5 months after junction.

"Early Disease"

Death of one partner occurred, occasionally, around the end of the 1st week. This was most common in mice of the BALB/c strain and occurred in both the presence and absence of genetic differences between partners. Gross and microscopic studies of these mice were inconclusive except for an occasional focus of myocardial inflammation and, in one instance, thrombosis of the left auricle.

Death of Both Partners

Death of both partners, occasionally described in the literature, was at times noted, but its frequency was in inverse proportion to the thoroughness of postoperative observation. Two or three daily checks did away with this phenomenon. Death of the second partner, in our opinion, was always due to the lethal effects of the dead partner on the live one and not due to mutual adverse immune responses.

DISCUSSION

The essence of our observations is as follows: Parabiotic compatibility is primarily dependent on identical alleles at the *H-2* locus. "Bilateral" incompatibility, *each* partner having an *H-2* allele not carried by the other, results in separation of partners during or after the 2d week of union. Separation is preceded by cessation of dye transmission (Evans Blue and Hypaque) from the 6th day on. Persistence of union beyond the usual time of separation, "false union," is merely passive and not accompanied by dye transmission. "Unilateral" incompatibility, one partner having an *H-2* allele not carried by the other, as is the case in many "pure F_1 hybrid" unions, usually results in "parabiotic intoxication" of the partner carrying the foreign allele. Death occurs most commonly during the last half of the 2d week or the first half of the 3d week. Dye transmission ceases early during the 2d week. Partners differing only in sex or at weak loci (*H-1* and *H-3*) usually remain in permanent union. In pairs differing at the *H–3* locus, Hypaque transmission also appears to be permanent. In pairs differing at the *H–1* locus, Hypaque transmission is consistent but has been noted as late as 10 months. In pairs differing only in sex, but of a strain in which females are known to reject male skin grafts, Hypaque transmission occasionally ceases after 1 month, but at times persists beyond 5 months.

It should be realized that cessation of Hypaque transmission does not necessarily indicate complete cessation of exchange of constituents between

partners. Bichel and Holm-Jensen have demonstrated (*4*) that P^{32}-labeled erythrocytes are transmitted as late as 7 weeks after union between reportedly unrelated mice, though to a lesser extent than between isologous partners. Our experiments with unilaterally incompatible mice, as discussed later, also suggest that even after cessation of Hypaque transmission, immune bodies may cross over into the hybrid partner. Cessation of Hypaque transmission is merely a convenient and sensitive method indicating host reactions of an intensity somewhat similar to those effecting rejection of skin grafts.

Splenic enlargement, with myeloid hyperplasia, anemia, or general malaise, that resulted in death was occasionally observed in pairs irrespective of their genetic background, but more frequently and intensely in genetic disparity. The question arose whether these symptoms were caused by this disparity. It seemed most likely, though, that they were occasioned by nonspecific events, such as infection, trauma, blood loss, or incidental disease, but aggravated by the genetic disparity. To test this interpretation, and on the assumption that it would simulate parabiotic union from an immunologic point of view, groups of mice received subcutaneous injections of incompatible spleen cells, daily, for 15 days. The mice developed neither anemia, general malaise, nor, on being killed, was there any evidence of splenic enlargement or myeloid hyperplasia.

Parabiotic Intoxication

The general dynamics of this phenomenon are readily understood as the result of an immune response of one partner to a second partner carrying a strong antigen lacking in the first; the second partner, carrying all antigens of the first and some of its own, does not act against the first partner. There are, however, several aspects that need clarification. One of them is the difference in behavior between unilaterally and bilaterally incompatible pairs. The former develop parabiotic intoxication, while the latter separate: One affects the hybrid partner systemically; the other is essentially local and affects the zone of injunction. Either may arise in response to the same antigen (A *vs.* B and A *vs.* AB), except that in the latter the genes determining the pertinent antigen(s) are heterozygous. Most likely both reactions take place in either case, more or less simultaneously, and in part offset each other. Sealing off the zone of junction, the result of the local reaction, interferes with the flow of cytotoxic products into the partner, particularly in bilaterally incompatible pairs in which each partner seals off from the other. In an occasional bilaterally incompatible pair the systemic reaction may be relatively strong and the local reaction relatively weak. Parabiotic intoxication of one or both partners would ensue. This situation may have prevailed in some of the bilaterally incompatible pairs (table 2) in which one partner died during the 2d or 3d week. Inversely, we have recently seen a few unilaterally incompatible pairs that survived beyond the usual period of intoxication and finally separated in a manner similar to bilaterally incom-

patible pairs. One could assume that in such instances the local reaction, though unilateral, was sufficiently strong to interfere with the systemic cytotoxic effect.

The nature of the anemia of the hybrid partner is not clear. Several explanations have been offered, and all are based on the assumption that immune bodies are produced by the pure partner, which affect the hybrid. One of these explanations is as follows: On establishment of adequate vascular anastomoses, erythrocytes from the hybrid enter the pure partner and are agglutinated, while erythrocytes of the pure partner are not agglutinated within the hybrid. The agglutinated erythrocytes, by virtue of their clump size, are prevented from returning to the hybrid; in consequence, the pure partner bleeds the hybrid dry. Although this assumption would explain, in part, the observed difference in erythrocyte counts between partners, other evidence favoring this concept has not been found.

There is another explanation, likewise lacking supportive evidence, which assumes that, in addition to direct vascular anastomoses between partners, there is vascular growth from each partner into the other—blood channels that permit both the inflow and outflow of blood to a limited area of the partner. In consequence, blood from one partner could circulate through a limited area of the other without utilizing the other partner's blood vessels. In due time, in the pure partner, the immune response would obliterate both direct vascular anastomoses and the hybrid vessels, while the hybrid, unable to obliterate the blood vessels of the pure partner, would remain exposed to the immune response of the pure, though direct vascular anastomoses between partners are already obliterated. The anemia of the hybrid, then, would be the result of the immune response on the hybrid partner. There could be either underproduction of cells, destruction of circulating cells, or both. However, the bone marrow of the hybrid at the time of death is morphologically normal, the reticuloendothelial apparatus shows no evidence of increased storage of blood pigment, and during the period of intoxication, the urine and feces of both partners lack excessive amounts of urobilinogen. Also, the serum bilirubin of either partner is not significantly elevated.

Both explanations offered fail to take into account that at the time of severe intoxication (2d week of union) exchange of constituents between partners has ceased, or is at least severely impeded, as our transmission studies with Evans Blue and Hypaque indicate. It is unlikely that antibodies having crossed over into the hybrid prior to the sealing-off period would make themselves increasingly felt during the week thereafter. One is then tempted to conclude that the immune effect is exerted by cells instead of circulating antibody, *i.e.*, cells which have crossed over into the hybrid prior to the sealing-off period have persisted within the confines of the hybrid and exert an immune effect comparable to that observed by Trentin (*5*), in young F_1 hybrids injected with cells of the parental strain. To test this possibility, we transferred suspensions of cells from the spleen and lymph nodes of pure partners that had been in parabiosis with hybrids

into other similar hybrids. However, recipients of such suspensions developed neither intoxication nor anemia. Further doubt of the crucial importance of lymph node and/or spleen cells in parabiotic intoxication is cast by the following observation: Transfer of lymph node or spleen cells from pure partners that had been in parabiosis with hybrids to other pure animals, prior to parabiosis with similar hybrids, does not affect the incidence, severity, or time of onset of intoxication.

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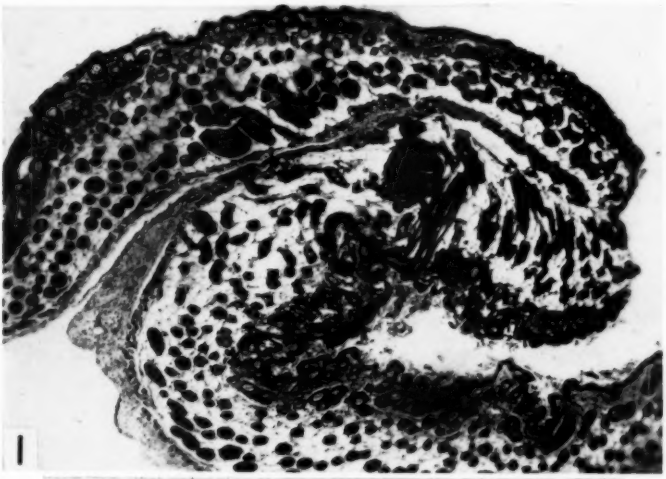
PLATE 117

FIGURE 1.—Junctional site on completion of parabiotic procedure: Skin edges are raised and held together by suture—round, dark mass *right* of *center*. *Note* panniculus carnosus of both partners raised with the skin, with resultant ample contact of subpannicular surfaces. Vacant space *left lower* corner is "inter-partner cavity." × 42

FIGURE 2.—Junctional site of incompatible partners, 14 days after union: Lucid vertical zone between partners extends from epidermis to interpartner cavity, and within lucid zone is a dark septum composed of degenerating neutrophils. × 42

FIGURE 3.—Junctional site of compatible partners, 14 days after union: Pannicular layers between partners have fused, interpartner cavity is obliterated, and zone of junction is characterized by a slender, vertical fibrous band. × 42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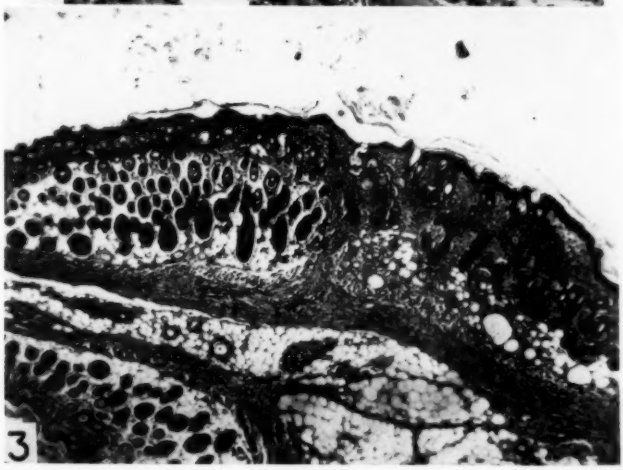
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PLATE 118

FIGURE 4.—Lack of transmission of Evans Blue between incompatible partners that have remained in state of "false union."

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PLATE 119

FIGURE 5.—Transmission of Hypaque in compatible partners or incompatible partners before 6th day of union. Shadow in neck of *left* partner indicates site of subcutaneous injection of dye.

FIGURE 6.—Nontransmission of Hypaque in incompatible partners after the 6th day of union. *Note* deposit of dye in neck of injected partner.

5

6

Changes in Deoxyribonucleic Acid Content of Rat Liver Cells During Ethionine Intoxication [1,2]

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SUMMARY

Changes in the deoxyribonucleic acid content of individual liver-cell nuclei were followed, during chronic ethionine intoxication, by Feulgen microspectrophotometry and the two-wavelength method. During ethionine intoxication, while substantially all liver cells are either diploid or tetraploid, the ratio of their numbers changes from 0.2 diploid cells per tetraploid cell in the normal animal to 2.6 diploid cells per tetraploid cell after 9 weeks' intoxication. The extensively proliferating interstitial (ductular) cells belong almost completely to the diploid category. The possible explanations of these changes are discussed.—J. Nat. Cancer Inst. 23: 1215–1225, 1959.

CHRONIC ETHIONINE intoxication causes conspicuous lesions in different organs, especially in the liver of rats, and may lead to hepatocellular cancer. After an initial stage of fatty metamorphosis, necrosis of single liver cells and regeneration are associated with extensive proliferation of interstitial cells, so-called ductular cells, and, probably, Kupffer's cells. Changes in the deoxyribonucleic acid (DNA) content of individual liver-cell nuclei were determined by Feulgen microspectrophotometry to study the features of the regeneration after single liver-cell necrosis characteristic of ethionine intoxication (*1–5*).

MATERIALS AND METHODS

From 60 DL-ethionine-fed [4] female Sprague-Dawley rats, 7 examples showing the most advanced histologic changes characteristic of ethionine intoxication were selected for microspectrophotometric measurements. The results of these measurements on these 7 livers indicated clearly the tendency of the changes. Therefore the number of animals selected for the measurements seemed to be adequate. The average initial body

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[2] Supported by grant C-3342 (C 1), Division of Research Grants, National Institutes of Health, Public Health Service.
[3] Postdoctoral Research Fellow, Public Health Service, on leave of absence from the Department of Pathology, University of Vienna, Austria.
[4] The compound used was a DL-ethionine obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. It was incorporated into the diet at a level of 5 gm. per kg. of diet.

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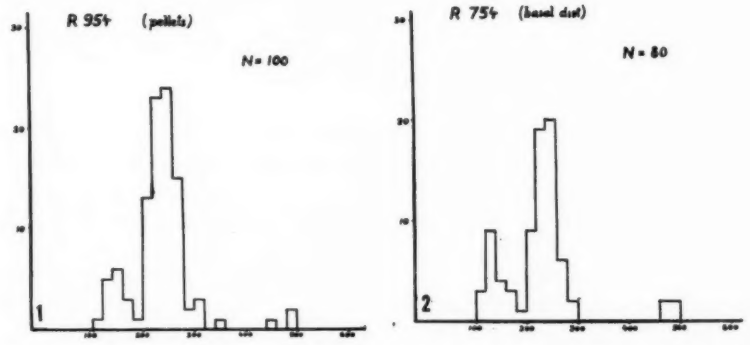
weight of the animals was 100 gm. The maintenance and the diet used were the same as that given by Singer *et al.* (*6*). Two animals on basal diet and one on stock diet served as controls. The final weights of the control animals were 140, 220, and 190 gm., respectively. The animals were killed by heart puncture and exsanguination under ether anesthesia, and pieces of the liver were fixed in Carnoy's solution for 4 to 6 hours, dehydrated, and embedded in paraffin. Sections from all livers were stained simultaneously under identical conditions. The random selection of nuclei with different diameters is influenced by the thickness of the section. Increasing the thickness of the section diminishes the proportion of cut nuclei and increases that of overlapping nuclei. Both cut and overlapping nuclei have to be excluded from the measurement. A thickness of 12 μ seemed to be the most adequate compromise for the measurement of liver-cell nuclei, and one of 8 μ seemed to be most suitable for the measurement of ductular-cell nuclei. The 8- and 12-μ-thick sections were hydrolyzed for 12 minutes in 1 N HCl at 60° C., placed in Schiff's reagent (deTomasi) for 60 minutes, and then rinsed in bisulfite, dehydrated, and mounted in synthetic liquid to match the refraction of the cytoplasm. (Cargille Refraction Liquids N_D 25° C.=1.574, R. P. Cargille Laboratory, New York.)

For the measurement of the Feulgen absorption expressed in arbitrary units, the 2-wavelength method (7–9) and the microspectrophotometer, as described by Pollister and Ornstein, (*10*) were used. The 2-wavelength method was chosen to avoid potential errors due to the inhomogeneous distribution of the material, and the microspectrophotometer was checked carefully for possible errors, in the usual manner. Liver-cell nuclei selected for the measurement fulfilled the following criteria: They were intact, were not overlapped by other nuclei, and had a diameter of less than 10 μ. The nuclei were selected regardless of their lobular position. No particular zonal differences could be observed in ethionine intoxication. Particular attention was paid to avoid the measurement of cut nuclei. In addition, the ratio of nuclei with a diameter of more than 10 μ to those with one less than 10 μ was determined with a projecting microscope, and the Feulgen absorption of some of the larger nuclei was measured with an eyepiece of lower magnification. This procedure seemed to be necessary because the measurement of 100 randomly selected intact nuclei cannot reflect the increased frequency of these larger nuclei when they occur in a frequency of less than 1 percent. This increase in the frequency, however, was suggested by the microscopic observation. For the measurement of the Feulgen absorption of ductular-cell nuclei, their size, their localization, and the absence of a nucleolus characteristic of liver cells served as guides. The possibility that some Kupffer-cell nuclei were measured as ductular-cell nuclei cannot be entirely excluded. Cells of questionable character were not measured.

RESULTS

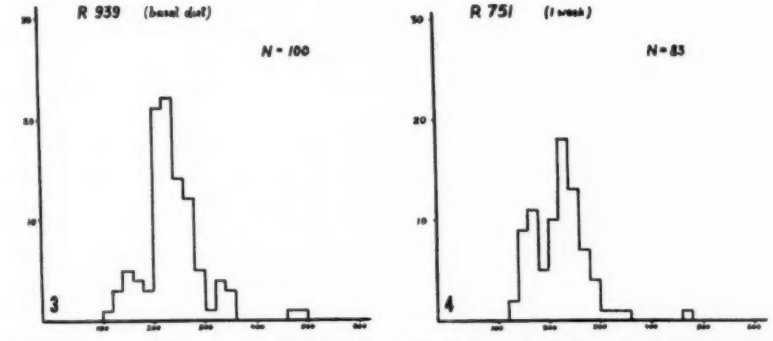
Normal Animals

The Feulgen absorption fell into 3 classes (text-figs. 1, 2, and 3): Class I, containing Fuelgen material of 0 to 200 units; Class II, of 200 to 400 units, and Class III, of 400 to 800 units. The frequency distribution was as follows: Class I, 16 percent in 2 animals and 25 percent in a 3d animal; Class II, 70, 81, and 82 percent; Class III, 2, 3, and 5 percent, respectively, in the 3 animals (table 1). The means of Feulgen values varied in Class I from 139 to 156; in Class II from 241 to 248; and in Class III from 474 to 479. The frequency of nuclei with diameter larger than 10 μ was less than 0.1 percent.



TEXT-FIGURE 1.—Frequency distribution of Feulgen absorption in liver cells f rat kept on stock diet.

TEXT-FIGURE 2.—Frequency distribution of Feulgen absorption in liver cells of rat kept on basal diet for 1 week.



TEXT-FIGURE 3.—Frequency distribution of Feulgen absorption in liver cells of rat kept on basal diet for 9 weeks.

TEXT-FIGURE 4—Frequency distribution of Feulgen absorption in liver cells of rat fed ethionine for 1 week.

TEXT-FIGURE 5.—Frequency distribution of Feulgen absorption in liver cells of rat fed ethionine for 2 weeks.

TEXT-FIGURE 6.—Frequency distribution of Feulgen absorption in liver cells of rat fed ethionine for 3 weeks.

Ethionine Intoxication

The frequency of nuclei of Class I was markedly increased, while that of Class II decreased (text-figs. 4 to 11). The percentage of nuclei of Class I after 1 week of ethionine feeding was 33 percent, and after 9 weeks, 72 percent, while the percentage of nuclei of Class II dropped from 66 to 28 percent. Nuclei of Class III with a diameter less than 10 μ were found and measured only in 3 animals, with about 1 percent frequency. The frequency of nuclei with a diameter larger than 10 μ rose during the intoxication, from 1 per 1,000 to 10 per 1,000. In 2 animals (R 863 and R 900), the Feulgen absorption of 5 each of the large nuclei was measured. The values fell into Class III. These values are not included in the table and are not shown in the frequency diagrams because the occurrence of these cells was less than 1 percent. The means of the values in Class I and Class II varied from 156 to 166 units and from 240 to 250 units, respectively. The coefficient of variation ranged from 9.5 to 16.1 percent (table 1). During the same period of ethionine intoxication, the mitotic index rose up to 7 mitoses per 1,000 cells. In the control animals the mitotic index was less than 1 per 10,000.

In one rat fed ethionine for 7 weeks, the Feulgen absorption in the extensively proliferated ductular cells was measured—98 percent of the values belonged to Class 1 and 2 percent to Class II. The mean of the Feulgen absorption in Class I was 122 units, coefficient of variation 22.3 percent (text-fig. 12). In normal rat livers measurable nuclei, which would correspond to the proliferating ductular cells, cannot be found in a number high enough to give statistical significance.

DISCUSSION

Ethionine is an antimetabolite to methionine. The mechanism of ethionine intoxication and the site of the methionine-ethionine antagonism

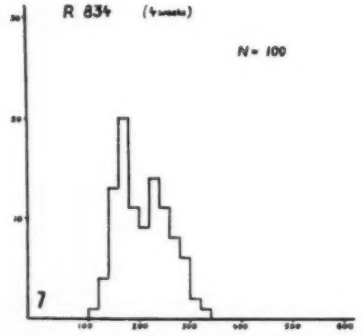
TABLE 1.—Distribution and changes of DNA content in individual liver cells during ethionine intoxication

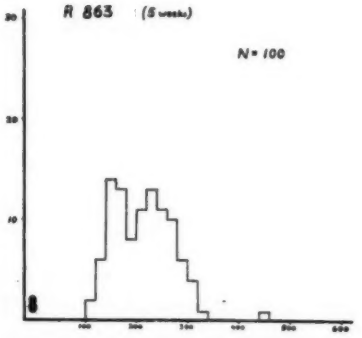
Number	Treatment	Class I* (0 to 200)	Class II* (200 to 400)	Class III* (400 to 800)	Large nuclei†	Mitoses/ 1,000 cells
R 954	Control (pellets)	16% 146. 0 (14. 0)	81% 246. 3 (10. 7)	3% 474. 0 (4. 0)	0	<1
R 754	Control (basal diet, 1 week)	25% 138. 8 (14. 1)	70% 241. 5 (8. 8)	5% 478. 5 (2. 8)	0	<1
R 939	Control (basal diet, 9 weeks)	16% 155. 7 (14. 9)	82% 248. 0 (11. 4)	2% 477. 5 (1. 6)	0	<1
R 751	Ethionine, 1 week	32. 5% 163. 9 (9. 9)	66. 3% 245. 6 (11. 8)	1. 2% 470. 0	1	2
R 788	Ethionine, 2 weeks	41. 7% 163. 3 (12. 7)	58. 3% 239. 7 (9. 8)	<1%	2	1
R 813	Ethionine, 3 weeks	48. 5% 166. 0 (11. 0)	51. 5% 242. 5 (9. 7)	<1%	3	3
R 834	Ethionine, 4 weeks	49% 164. 8 (12. 5)	51% 247. 8 (11. 5)	<1%	4	4
R 863	Ethionine, 5 weeks	43% 160. 4 (14. 5)	56% 249. 0 (12. 4)	1% 458. 0	6	5
R 900	Ethionine, 7 weeks	61. 5% 157. 2 (14. 2)	37. 5% 248. 7 (12. 2)	1% 472. 0	5	7
R 900‡	Ethionine, 7 weeks	98% 122. 4 (22. 3)	2% 249. 5			
R 936	Ethionine, 9 weeks	72% 156. 4 (16. 1)	28% 249. 8 (9. 5)	<1%	10	5
Mean§		157. 2 (5. 9)‖	245. 9 (1. 5)‖	471. 7 (1. 7)‖		

*The numbers are: frequency, mean, and coefficient of variation in parentheses.
†Number of nuclei with a diameter larger than 10 μ per 1,000 liver cells.
‡Ductular cells.
§Mean excluding ductular cells.
‖Coefficient of variation of the mean values.

have not been established. However, alterations of protein synthesis have been assumed. This is morphologically reflected in single cell necrosis of the liver cells and in proliferation of ductular cells—the latter are inhibited by administration of cortisone (*11*). The extensive proliferation of ductular cells, which parallels the enlargement of the liver, may explain the increase in DNA concentration found chemically in subacute ethionine intoxication (*12*).

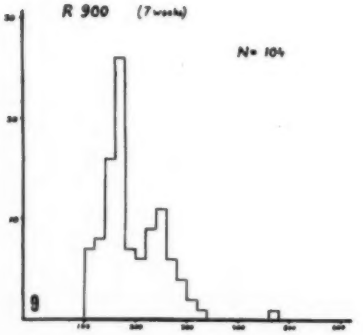
Feulgen microspectrophotometric measurements indicate that ethionine feeding leads to an increase of the frequency of cells of Class I, probably diploid cells, while the frequency of cells of Class II, probably tetraploid cells, decreases. The frequency of the Class III nuclei does

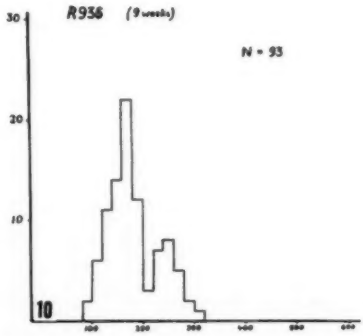




TEXT-FIGURE 7.—Frequency distribution of Feulgen absorption in liver cells of rat fed ethionine for 4 weeks.

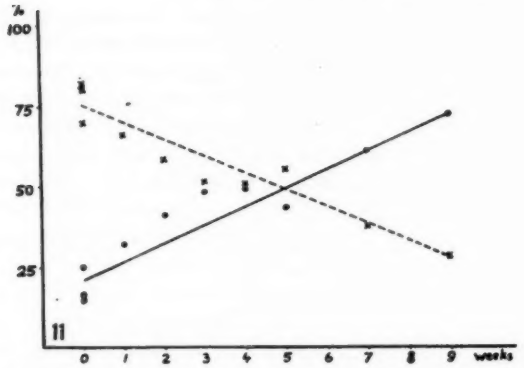
TEXT-FIGURE 8.—Frequency distribution of Feulgen absorption in liver cells of rat fed ethionine for 5 weeks.



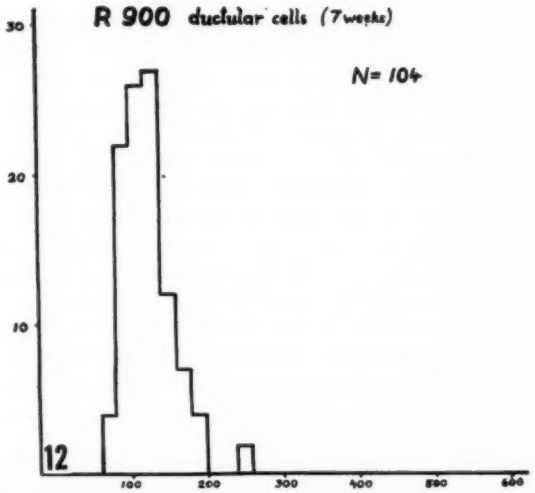


TEXT-FIGURE 9.—Frequency distribution of Feulgen absorption in liver cells of rat fed ethionine for 7 weeks.

TEXT-FIGURE 10.—Frequency distribution of Feulgen absorption in liver cells of rat fed ethionine for 9 weeks.



TEXT-FIGURE 11.—Percentage changes of diploid and tetraploid cells in relation to ethionine feeding. Values at *zero* week represent the percentages of diploid and tetraploid cells found in control animals. *Solid line:* diploid cells; *broken line:* tetraploid cells.



TEXT-FIGURE 12.—Frequency distribution of Feulgen absorption in ductular cells of rat fed ethionine for 7 weeks.

not increase, but nuclei with a larger diameter can be observed in greater number. These, however, seem to contain DNA corresponding to that of Class III nuclei. This indicates that the volume of Class III nuclei and the concentration of DNA are influenced rather than the amount of DNA per nucleus. During the experimental period, which is too short to develop cancer, no cells were found with amounts of Feulgen material suggesting higher polyploidy than octoploidy. Even the largest cells measured, which increased up to 10 per 1,000, contained amounts of DNA corresponding to octoploid cells. These changes were associated

with a marked increase of the mitotic rate. However, this was not reflected in significant alterations of variation and means of DNA values in Class I and Class II nuclei when compared with control animals.

During postnatal development the frequency of diploid cells of rat liver decreases, while the frequency of tetraploid cells increases (*13*, *14*); but during regeneration after partial hepatectomy, the percentage of diploid cells decreases and the frequency of octoploid cells increases (*15*). Thus it appears that the DNA pattern of liver cells in ethionine intoxication resembles that of young rats rather than that of partially hepatectomized rats.

The shift of the proportion of tetraploid cells to diploid cells in ethionine intoxication could reflect a greater sensitivity of tetraploid cells to the injury causing their partial disappearance. More probable is an alternative explanation, that the regenerating cells, as the stem cells of the liver, are diploid cells, and extensive regeneration is followed by an increase in the frequency of diploid cells. This regeneration is apparently different from that seen after partial hepatectomy, which restores the original structure of the liver.

In early postnatal development the rat liver shows a high mitotic rate which drops sharply after the 9th week (*13*, 14). In the adult rat liver the mitotic rate is extremely low, probably less than 1 per 10,000 cells. One can assume that the decrease of the mitotic rate is related to the reduction of the frequency of diploid cells and to the increase of that of the tetraploid cells mentioned previously. The decrease of the mitotic rate has been correlated with the development of a highly specialized parenchyma in the sense of antagonism between mitosis and cytoplasmic differentiation (*16*). It seems reasonable to assume that the cytoplasmic mechanism of cell division is inhibited by the specialized function of the liver cell, but the DNA synthesis is not. This could explain the low mitotic rate as well as the increase of the frequency of tetraploid cells. In ethionine intoxication single liver cells become necrotic and concurrently the specialized cellular functions of other cells are suppressed, resulting in a high mitotic rate (table 1) and an increase of the frequency of diploid cells.

During the ethionine intoxication a greater number of larger nuclei with a diameter over 10 μ was observed. These nuclei seemed to belong to Class III, according to their DNA values. However, the frequency of Class III nuclei including even these large nuclei was not increased in comparison with the control animals. This means that the volume of these large nuclei was increased, but not the frequency of Class III nuclei. The frequency of these cells was relatively low (maximum 10 per 1,000), and therefore it was rather difficult to get measurements on a number of them high enough for statistical significance.

The Feulgen absorption of ductular-cell nuclei was measured in one animal. The results of these measurements indicated that almost all ductular-cell nuclei measured belonged to Class I. Technically it is difficult to measure the ductular cells, since they are densely packed and

overlapping. Conclusions concerning the origin and fate of these ductular cells would not be justified from measurements in only one animal.

The results of the present investigations have been discussed under the tacit assumption that Classes I, II, and III correspond to diploid, tetraploid, and octoploid cells, respectively. The first question is whether nuclei grouped in one class actually represent a biological class of individual cells or, in other words, whether the variation found within one class can be reconciled with the theory of DNA constancy. The second question concerns the value of 1.56 of the ratio of the means of Class II and Class I instead of the theoretical 2.0, if Class II represents tetraploid cells and Class I, diploid.

Lison and Valeri (*17*) have concluded that the variation of DNA values of diploid cells and binucleate cells is too high and does not support the constancy theory. If it is accepted that the stem cell of the liver is diploid and the diploid cells are undergoing transformation in the direction of tetraploid cells, the variation, which ranges up to 16 percent in the present study, is not contrary to the constancy theory. It may be simply an expression of DNA synthesis associated with the transformation of diploid into tetraploid cells—a DNA synthesis without cell division.

The same reasoning may explain that a ratio different from the theoretical one has been found between the means of Class I and Class II cells. The ratio between the amount of DNA in the diploid and tetraploid cells has been reported to be near the theoretical 2 (*18–23*; except Naora *24*). In the present study the ratio of the means of Class II to Class I was 1.56, while the ratio of the means of Class II to Class I of the ductular cells was 2.01. The ratio much lower than the theoretical one might be caused by technical errors. This was contradicted by the low variation of Class II values and the perfect ratio obtained for the ductular cells, though the nuclei of Class II were larger and the possibility that cut nuclei were measured was higher. The values of Class II probably correspond to a tetraploid chromosome set, half of which, found in ductular cells, corresponds to the true diploid value; the higher values of liver cells found in Class I are expressions of DNA synthesizing mechanisms discussed previously. The mean values of Class I therefore reflect the age of the animal, the ratio of regeneration, and of the process leading to formation of tetraploid cells. However, the possibility that other physiologic or pathologic factors contribute to the variation found in these classes cannot be excluded. The method used in this study is probably not sensitive enough to reveal these alterations.

ADDENDUM

In 2 animals fed, for 5 weeks, a diet containing 0.75 percent ethionine and supplemented with 0.6 and 1.2 percent methionine, respectively, the following distributions of DNA values were found: *No. 1.* Class I: 11.3 percent, Class II: 88.7 percent, Class III: less than 1/62; *No. 2.* Class

I: 28.3 percent, Class II: 71.7 percent, Class III: less than 1/60. (The DNA values in control and ethionine-fed animals of this experimental series are in accordance with those reported.) The results support the conclusions, based on histological observations (Stein *et al.*: Proc. Soc. Exper. Biol. & Med.: Effect of Methionine upon Ethionine Intoxication on the Rat; in press), that methionine dissociates the interstitial and liver-cell reactions in ethionine intoxication. The ductular-cell reaction is prevented, and the liver-cell damage is suppressed.

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Mammogenesis and Breast Carcinogenesis in Virgin Female Mice of BALB/c$_f$ Substrain With the Milk Agent [1, 2]

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SUMMARY

A morphologic study on the mammary glands of virgin female mice of the BALB/c$_f$ substrain with the milk agent has shown that alveolar development takes place from the end of the 3d month of life until the senile involutional changes affect the end pieces of the glandular tree early in the 2d year. Small clusters of alveoli or small nodules are recognizable by the 4th month and progressively increase in number until they reach a peak at the 10th month. Some of them progress toward large nodules, but the others regress in connection with the senile changes. Large nodules, which appear by the 6th month, show a moderate numerical increase in direct relation to the age of the animal. Some of them progress to cancer, but others do not escape the senile involution. Palpable mammary tumors appear from the 7th month, but no tumors have been observed in females that live longer than 15 months. With the breast development and regression as detectors of the hormonal stimuli, it should be supposed that, in virgin females of the BALB/c$_f$ substrain, the mammogenic triad (estrogens + progesterone + prolactin) is effective on the breasts, but only from the 4th month to the end of the 1st year. After this time there is evidence of a lack, or deficiency, of hormonal stimuli, which seems responsible for the senile involutional changes, for the disappearance of nodules, and for the nonoccurrence of mammary tumors in old animals. Results obtained in this substrain cannot be generalized to other strains.—J. Nat. Cancer Inst. 23: 1227–1238, 1959.

NORMAL GROWTH of the mammary glandular tree is caused by the female sex hormones. It is accepted that estrogens promote the development of the duct system, and progesterone promotes that of the lobulo-alveolar system (*1*). Estrogens and progesterone act on the breast only when the hypophysis is present (*2*). Mammogenic activity of the hypophysis seems to be carried out by prolactin, since estrogens, progesterone, and prolactin induce development of the mammary glandular tree with lobulo-alveolar differentiation in ovariectomized, adrenalectomized, and hypophysectomized animals. However, when the growth hormone is added to this hormonal triad, the mammary response is increased (*3*).

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[2] This investigation has been supported by research grant C-3844(R1) from the National Cancer Institute, National Institutes of Health, Public Health Service.

In female mice, after the onset of sexual cycles, a rhythmic and progressive development of the mammary-duct system occurs. During proestrus the ducts are thin and show a few solid, lightly stained buds. At estrus the buds develop new ducts and other buds appear. At metestrus the glands undergo slight retrogressive changes. However, ductal proliferation resumes during the next estrous cycle. Therefore with each succeeding estrous cycle the growth of the ducts is slightly extended until the complete development is reached (*4*). The lack of a rhythmic development of alveoli at metestrus, *i.e.*, the time in which estrual corpora lutea are formed in the ovaries, has led some investigators to suppose that the sexual cycle of mice is characterized by a defect of luteal hormone (*1*), or that the estrual corpora lutea do not function in mice (*5*).

Nevertheless, in the breasts of virgin female mice that carry the milk agent, circumscribed proliferation of alveoli, the alveolar hyperplastic nodules that are precancerous, occurs some months after birth. The nodules are clusters of alveoli indistinguishable morphologically, histochemically, and biochemically from the lobules of pregnancy (*6–8*). The luteal hormone seems to be an important factor in the etiology of nodules and of tumors arising in nodules. The question arises as to the source(s) of progesterone in virgin female mice.

The alveolar nodules are supposed to be, at least in part, independent of the hormonal stimuli (*9*, *10*). Some of them increase in size progressively and, after a certain time, tumors grow by progression. However, it is known that mammary tumors do not develop in old virgin female mice of some high-cancer strains (*11*). This might be due to a progressive defect of sex hormones in old animals, though it has been shown that a true "menopause" does not occur in mice (*12*). The question arises as to the fate of nodules which do not progress to cancer and the importance of sex hormones in the progression of nodules.

To answer some of these conflicting questions, a morphologic study of the mammary glands, from birth to later life, was undertaken in virgin female mice of the BALB/c_f substrain with the milk agent.[3]

The milk agent is known to influence the development of the mammary glandular tree, as well as the occurrence of mammary nodules and tumors. However, it is considered a nonessential factor in breast carcinogenesis, since nodules and tumors may also be observed, though fewer and later, in susceptible mice deprived of the agent by foster nursing (*5*). This paper deals mainly with hormones in the development of the mammary glandular tree, hyperplastic alveolar nodules, and mammary tumors in susceptible, agent-bearing mice.

MATERIALS AND METHODS

Sixty-seven virgin female mice of the BALB/c_f substrain were killed with ether at monthly intervals, 1 to 8 each month, from the 1st to the

[3] BALB/c foster-nursed by C3H (BALB/c_fC3H/Cb/Se substrain) sent from the Chester Beatty Research Institute to Professor Severi, November 6, 1953.

20th month of life, with the exception of the 12th month. The right or left mammary gland of the second thoracic pair was removed from each animal and prepared by the wholemount method. Four of these animals developed palpable breast tumors at 10, 11, 14, and 15 months of age.

The wholemounts of breast tissue were examined with a Reichert Lanameter for the presence of buds, small terminal branches, isolated alveoli, and clusters of alveoli or nodules. Alveolar clusters were also measured in the two largest diameters, recorded when their mean diameter was higher than 0.1 mm., and then divided into the following classes of size: $>0.1 \leq 0.2$; $>0.2 \leq 0.3$; $>0.3 \leq 0.4$; > 0.4 mm.

Throughout their lives, 73 virgins and 229 breeding females of the BALB/c_f substrain were kept under observation for the appearance of palpable mammary tumors. Fifteen virgin females and 52 intact males, treated with weekly subcutaneous injections of 0.05 mg. of estradiol benzoate in olive oil, were observed for tumor incidence and tumor age. Palpable tumors were examined histologically by the routine methods.

All animals were kept in metallic cages, 2 to 4 in each cage, fed with pellets, and given water *ad libitum*.

RESULTS

Tumor Incidence and Tumor Age Under Different Hormonal Conditions

In table 1 the tumor incidence and tumor age in virgin, breeding, and estrogenized females and males of the BALB/c_f substrain are shown. Thirty-seven percent of virgins developed one or more mammary tumors at an average age of 309 days, compared with 81.2 percent of breeding females that grew tumors at an average age of 218 days. In this substrain, pregnancy doubles the percentage of tumors and lowers the tumor age.

Tumor incidence in virgins of the high-mammary-cancer strains is controlled by the inherited hormonal influence. In the strains that possess the hormonal influence, *e.g.*, the C3H strain, virgin females have

TABLE 1.—Tumor incidence and tumor age in virgin, breeding, and estrogenized females and males of BALB/c_f substrain

BALB/c_f substrain	Number of mice	With mammary tumors: Number	Percent	Average age of tumors (*days*)	Without tumors: Number	Average age at death (*days*)
Virgin females	73	27	37.0	309	46	357
Breeding females	229	186	81.2	218	43	252
Virgin females treated with estradiol benzoate	15	15	100.0	158	0	—
Intact males treated with estradiol benzoate	52	42	80.7	288	10	300

a high-mammary-tumor incidence; they also show more and larger corpora lutea in the ovaries (*13*) and, when gonadectomized, usually undergo adrenal hypertrophy. The hypertrophic adrenals may also assume a vicarious ovarian function and cause hyperplastic nodules and tumors in the breasts (*14*). By contrast, in the strains that do not possess the hormonal influence, *e.g.*, the A strain, virgin mice have a low-mammary-tumor incidence, show less and smaller corpora lutea, and do not undergo adrenal hypertrophy when they are gonadectomized. These data lead one to suppose that the inherited hormonal influence acts by increasing the production and/or the release of gonadotrophic hormones by the pituitary (*15*).

In respect to their tumor incidence, virgin females of the BALB/c_t substrain are in a mid-position compared with animals that possess or do not possess the hormonal influence. This should mean that a certain level of endogenous production of progesterone and prolactin is reached, in virgins of this substrain, which produces a percentage of tumors about midway between.

The same effect of pregnancy in increasing tumor incidence and in decreasing tumor age is carried out by the administration of estradiol benzoate, since 100 percent of virgin females and 80.7 percent of intact males, which received 0.05 mg. of estradiol benzoate in olive oil by weekly injections, developed mammary tumors (*16*). The administration of estradiol benzoate to intact females and males of this substrain leads at first to an intense ductal and lobulo-alveolar development in the mammary glands, which ends with tumor growth (*17*). This means that the administered estrogens induce a release of endogenous progesterone and prolactin in mice.

Mammary Development and Regression

The progressive and regressive changes of the mammary glandular tree in virgin females of the BALB/c_t substrain are summarized in table 2. Mammogenesis proceeds from the buds; each bud develops branches; terminal branches grow alveoli; and when the alveolar development is advanced, rudimentary lobules appear in the distal ends of the branches. In the table, the presence and gross quantity of buds, branches, and alveoli are recorded separately for each breast.

The most important features shown by the whole breasts of BALB/c_t virgins are: (1) the presence of alveoli from the 3d month, and (2) the senile involutional changes which affect the end pieces of the glandular tree early in the 2d year.

End and lateral buds are frequently seen in the breasts from the first months up to the 7th and 8th months, but disappear later. The small terminal branches, recognizable from the 3d month, undergo variations in number and size from animal to animal, until they progressively decrease in old mice. The earliest alveoli appear in the breasts at the end of the 3d month. They also undergo quantitative variations from animal to

TABLE 2.—Proliferative and regressive physiologic changes and number of alveolar hyperplastic nodules in mammary glands of BALB/c$_f$ virgin female mice of scalar ages*

Age (*months*)	1	1	2	3	3	4	4	4	4	5	5	5	5	5	5	6	6	7	7	7
Buds	+−−	+−−	+−−	+−−	++−	++−	++−	++−	+−−	+−−	+−−	++−	++−	+−−	+−−	+++	++−	++−	+−−	++−
Branches	−−−	−−−	−−−	++−	+−−	+−−	+−−	+−−	++−	+−−	+−−	+−−	+−−	++−	+−−	++−	++−	+−−	+++	+−−
Alveoli	−−−	−−−	−−−	+−−	−−−	+−−	+−−	−−−	++−	+−−	+−−	+−−	−−−	−−−	−−−	+++	+++	+++	+++	++−
Nodules†	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	8	0

Age (*months*)	7	7	7	7	7	8	8	8	8	8	9	9	9	9	9	9	9	10	10	10
Buds	−−−	+−−	+−−	+−−	+−−	−−−	+−−	−−−	−−−	+−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−
Branches	++−	+−−	++−	++−	+−−	++−	++−	++−	++−	++−	+++	+++	+−−	++−	++−	++−	+++	+++	++−	++−
Alveoli	+++	+−−	++−	+−−	+−−	+−−	++−	+−−	+++	+++	+++	+++	+−−	+−−	+−−	++−	+++	+++	++−	++−
Nodules†	2	0	0	0	0	1	0	0	1	0	0	3	0	1	0	0	6	27	1	4

Age (*months*)	11	11	13	13	14	14	14	14	14	15	15	15	16	16	16	16	17	17	17	17
Buds	−−−	−−−	R‡	R	R	R	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	R	−−−
Branches	++−	+++	R	R	R	R	+−−	−−−	−−−	−−−	−−−	−−−	++−	+−−	+−−	+−−	++−	+−−	R	−−−
Alveoli	++−	+++	R	R	R	R	−−−	−−−	−−−	−−−	−−−	−−−	+−−	−−−	−−−	−−−	+−−	−−−	R	−−−
Nodules†	3	17	3	23	9	5	0	0	0	0	0	0	1	0	0	0	0	0	0	0

Age (*months*)	18	18	18	19	19	19	20
Buds	R	−−−	−−−	R	−−−	−−−	−−−
Branches	R	−−−	+−−	R	++−	−−−	+−−
Alveoli	R	−−−	+−−	R	+−−	−−−	−−−
Nodules†	0	0	0	0	1	0	0

*Sixty-seven virgin females BALB/cf; thoracic mammary glands of the 2d pair; wholemount.
†Mean diameter = >0.4 mm.
‡R = in regression.

animal. Usually the increase in the number of alveoli shows a direct relationship with the animal's age. In several instances they are grouped to compose lobular and/or nodular clusters. The presence of alveoli should mean that progesterone and prolactin stimulate the breast tissue in virgin females of this substrain. Whether or not cyclic variations in the number of alveoli take place with the recurring estrous cycles still remains unanswered and is not included in this investigation. However, the first estrous cycles occur in the BALB/c_f substrain at the beginning of the 2d month (*18*), while the first alveoli occur in the breasts at the end of the 3d month. Therefore several estrous cycles occur in the early life of the mice without any alveolar development in the mammary glands.

Early in the 2d year, the breast tissue undergoes senile involutional changes. These affect mainly the alveoli and small terminal branches, while the gross duct system is unaffected. Morphologically, the breast involutions appear as a fragmentation of the glandular structures, which lose their color, and then disappear into the surrounding fat tissue. The picture is somewhat similar to that after pregnancy and lactation. This leads to the supposition that the senile involutional changes are due to a critical reduction of the hormonal stimuli on the breasts. As a result of the involution, the mammary glandular tree of old BALB/c_f virgin female mice is almost constantly of a pure ductal type. Nevertheless, estrous cycles still occur in old animals of all strains of mice investigated till now, though the cycles show irregularities and interruptions (*12, 19, 20*).

The preceding data lead to the conclusion that in virgin females of the BALB/c_f substrain there is a deficient liberation of progesterone and prolactin in the first and in the last months of life, while during middle age these hormones are produced in sufficient quantities to induce lobulo-alveolar development in the breasts.

Pseudopregnancy has been suspected of occurring in mice, independently of sterile coitus, because of crowding of females in the cages or even for other reasons. It cannot be excluded that the presence of alveoli and lobules in the breasts of the virgin mice is due to the occurrence of "spontaneous" pseudopregnancies. However, an investigation of the behavior of estrous cycles in BALB/c_f virgins, varying from 2 to 5 in each cage, failed to demonstrate spontaneous pseudopregnancies (*21*).

Hyperplastic Alveolar Nodules

The hyperplastic alveolar nodules are composed of grouped alveoli, which range from few to several hundreds, that are usually not cystic and are surrounded by a moderately increased layer of connective tissue without any appreciable evidence of inflammatory reaction (*22*). On the basis of this definition it appears difficult to recognize a nodule from a mammary lobule. In addition, there is no mention of what size a cluster of alveoli should be, to be considered a nodule.

In this investigation alveolar clusters have been measured in the two largest diameters and recorded when their mean diameter was higher

than 0.1 mm. Over this diameter the term "nodule" has been used, irrespective of the size of clusters, though perhaps the term is inappropriate for the small ones.

Table 3 shows the nodules divided according to their size and the age of the animal. Figures illustrate the average number of nodules per mammary gland. In table 2, nodules larger than 0.4 mm. are recorded for each breast.

Small nodules are recognizable at the 4th month, then progressively increase in number, reach a peak at the 10th month, and soon disappear almost completely. Some small nodules seemingly progress toward large nodules by an increase in their size, but most of them undergo involution in connection with the senile changes of the breast, as evidenced by the morphologic pictures (*21*).

Large nodules, which appear later and then disappear, show a moderate numerical increase in direct relationship to the age of the animal. Some of the largest nodules progress to cancer, but eventually several of them do not seem to escape the senile involution, since only 2 large nodules have been discovered in 21 mammary glands of different virgin females 14 to 20 months old (table 2).

The preceding data lead to the supposition that the hormonal dependence of nodules is a function of their size, but that nodules seldom reach a state of complete independence of the hormonal stimuli unless they undergo malignant degeneration by progression. Obviously, this conclusion is limited to the virgin females of the substrain investigated.

Mammary Tumors

Of 73 virgin females kept under observation throughout their lives, 27 developed 1 or more palpable tumors. The age distribution of tumors is illustrated in table 4. All tumors occurred between the 7th and the 15th month. No tumors have been observed in 8 females living longer than 15 months, while, for an even distribution, at least 3 tumors should have been discovered in these animals.

The nonoccurrence of tumors, after the 15th month, in virgin females of the BALB/c_t substrain appears closely related to the absence of nodules, which are the usual sources of tumor development. The absence of tumors seems indirectly connected with the deficiency, or lack, of the hormonal stimuli on the mammary glands.

Twenty-five of the 27 primary palpable tumors grown in these animals have been examined histologically and grouped according to the classification of Dunn (*23*) and modified by Olivi *et al.* (*24*). A marked prevalence of adenocarcinomas of the B type (17) compared to adenocarcinomas of the A (5), M (3), and C (0) types, has been observed.

DISCUSSION

The results of this investigation are schematically summarized in text-figure 1. Generalizations of these data must be avoided, since there are

TABLE 3.—Average number of nodules per mammary gland, according to diameter and animal age*

Animal age (*months*)	4	5	6	7	8	9	10	11	13	14	15	16	17	18	19	20	Total of averages	Total of counted nodules
Average diameter of nodules:																		
>0.1 ≤ 0.2 mm.	3. 5	1. 8	10. 5	5. 4	10. 2	18	44. 7	16. 5	4. 5	3. 8	0. 0	2. 5	1. 7	2	1	0. 0	126. 1	486
>0.2 ≤ 0.3 mm.	2. 5	1. 5	11. 5	7. 6	5. 2	9	45. 7	31	13. 5	7. 8	. 0	1. 5	. 0	1. 7	1	. 0	139. 5	475
>0.3 ≤ 0.4 mm.	. 2	. 2	1. 5	1. 2	1	2. 1	7. 3	16	8. 5	1. 4	. 0	. 2	. 0	. 0	. 0	. 0	39. 6	114
>0.4 mm.	. 0	. 0	2	1. 2	. 4	1. 4	10. 7	10	13	2. 8	. 0	. 2	. 0	. 0	. 3	. 0	42. 0	120†

*Sixty-seven virgin female BALB/c_f; thoracic mammary glands of the 2d pair; wholemount.

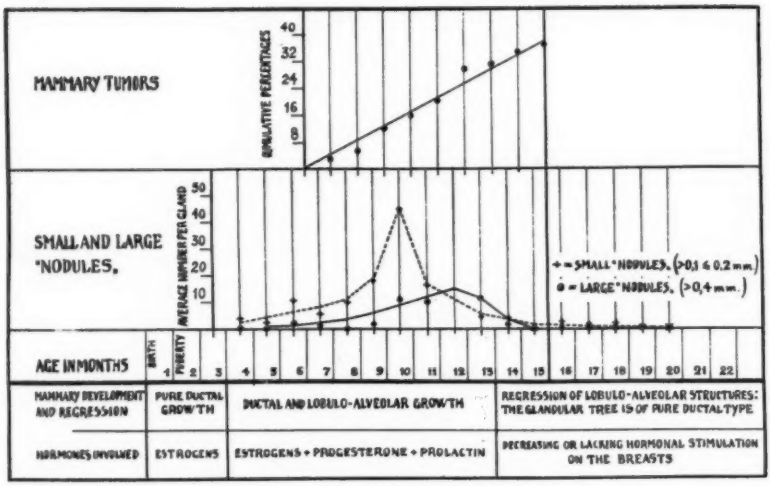
†Nodules of more than 0.4 mm. in diameter were grouped in the following classes of size:

Average diameter:	$\leq .5$	$\leq .6$	$\leq .7$	$\leq .8$	$\leq .9$	$\leq 1.$	≤ 1.1	≤ 1.2	≤ 1.3	≤ 1.4	≤ 1.5	≤ 1.6	$= 3.5$	$= 4$ mm.
Total nodules counted:	61	16	15	8	5	4	1	4	3	0	0	1	1	1

TABLE 4.—Age distribution of mammary tumors in virgin females of BALB/c_f substrain

Age (*months*)	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Total	Average age (*days*)
Number of mice with tumor	2	2	5	3	3	7	1	3	1	0	0	0	0	0	0	0	27	309*
Number of mice without tumor	2	4	6	3	5	10	3	3	2	1	2	1	1	0	1	2	46	357†
Total	4	6	11	6	8	17	4	6	3	1	2	1	1	0	1	2	73	—
Cumulative percentage of mice with tumor	2.7	5.4	12.2	16.3	20.4	30.0	31.4	35.5	37.0	—	—	—	—	—	—	—	37	

*Of tumor appearance.
†Of animal death.



TEXT-FIGURE 1.—Mammogenesis and breast carcinogenesis in BALB/c_t virgin female mice. Schematic representation.

no reasons at present to think that results obtained in this substrain can apply to other strains of mice.

In the second thoracic glands of the BALB/c_t virgin females, a very slow development of the large duct system during the first month of life can be seen. In the 2d month, as soon as the estrous cycles occur, duct development becomes more active, and the glandular tree extends itself into the surrounding fat pad by progressive ramifications. During the 3d month, small terminal branches appear, and ultimately alveolar development takes place along them. Initially there are few isolated alveoli, but early in the 4th month small alveolar clusters or lobules or nodules appear. After this time the number of alveoli and small nodules increases progressively until the end of the 1st year. At the 6th month, large nodules can be seen in the breasts; the number of these nodules becomes greater month by month, with a peak in the 13th month. During the 7th month, the first mammary tumors are discovered by palpation, and more palpable growths appear in the following months until all tumors have appeared at the 15th month.

Senile involutional changes take place in the breast early in the 2d year and require some months for their completion. Isolated alveoli, small nodules, and terminal branches are the first structures involved in regression, but eventually the large nodules that did not progress to cancer are affected. As a result, no breast tumors develop in old animals.

With breast development and regression as detectors of the hormonal stimulations on the glands, it should be supposed that, in virgin females of the BALB/c_t substrain, the mammogenic triad (estrogens + progesterone + prolactin) is effective in the mammary glands, but only for a

limited period of the animal's life, namely, from the 4th month to the end of the 1st year. After this time there is evidence of a lack or a deficiency of hormonal stimuli, which seems responsible for the senile involutional changes, for the disappearance of nodules, and for the nonoccurrence of mammary tumors in old animals.

According to the behavior of the mammary glands, the ineffectiveness of estral corpora lutea throughout the life of the animal, postulated for other strains of mice (*1*, *5*), cannot be accepted in this study. Also the question of nonoccurrence of menopause in old mice, which has been shown for other strains (*12*, *19*, *20*), should be reinvestigated in this substrain, since the breasts behave as if a menopause would occur. Another interesting feature to be stressed here, which is in contrast to previous reports in the literature (*9*, *10*), is the hormonal dependence of almost all the alveolar nodules, including the largest and well-established ones, before their malignant degeneration.

However, it must be emphasized that results obtained in virgins of the BALB/c_t substrain cannot be generalized. A recent investigation undertaken in old virgins of the RIII substrain has shown, for instance, that nodules are still numerous in the breasts beyond the latest cancer ages (*11*).

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A Graded-Response Assay for the Friend Leukemia Virus [1]

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SUMMARY

In Swiss mice inoculated intravenously with the Friend mouse leukemia virus, the mean log spleen weight at an arbitrary time after infection was a function of log virus dose. The dose-response function was S-shaped, with an upper shoulder or a plateau at high doses of virus, a linear phase, and a lower plateau with low doses of virus. Fitting of regression lines to the linear phase and calculation of the virus dosage corresponding to spleen-weight response of 500 mg. permitted reproducible quantitation of the agent. This procedure permits reasonable estimates of virus titer to be made on the basis of spleen-weight response to a single dilution of virus.—J. Nat. Cancer Inst. 23: 1239–1248, 1959.

FOR CHARACTERIZATION and study of a viral agent, procedures for quantitation of viral infectivity are of great importance. When existing techniques for working with an agent do not provide endpoints based on counts of infective centers, such as plaque or pock counts, extinction dilution titrations are generally used for quantitation. However, there are certain instances in which a relatively predictable quantitative relationship exists between dilution of virus inoculated and a precisely measurable, continuously varying parameter of infection; when such a relationship exists, quantitation can be carried out by graded-response functions, and a comparable or higher degree of precision can be obtained per experimental animal than in extinction dilution titrations. The graded-response assays reported for viruses, generally have been based on incubation period to appearance of disease in animals or to onset of cytopathic effects in tissue cultures (*1–7*).

Friend (*8*) has described a virus of mice which produces neoplastic proliferation of splenic reticulum cells, with hyperplasia of lymphocytic and erythroid elements (*9*). Since the predominant lesion in the gross is

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splenomegaly, an attempt was made to develop a bioassay procedure for quantitation of the agent, with spleen weight as a readily measurable parameter of infection.

MATERIALS AND METHODS

Mice.—Weanling (14 to 16 gm.) mice of the "General Purpose" non-inbred Swiss mouse strain from the National Institutes of Health Animal Production Colony were used. They were maintained in glass jars, 5 per jar, and fed Purina laboratory chow, small, with water *ad libitum*.

Virus.—Mice infected with the Friend agent were received from Dr. Charlotte Friend, Sloan-Kettering Institute for Cancer Research. The virus was maintained in mice by passage, at weekly intervals, of clarified 10 percent spleen suspensions, or filtrates, inoculated intravenously. Virus used in the experiments described here was in the seventh through 26th serial passage in this laboratory.

At various passage levels, large pools of virus were prepared by inoculating mice and harvesting spleens 1 or 2 weeks after inoculation. The spleens were homogenized in a chilled Waring blendor and suspended to 10 percent by weight in Hanks' solution containing 125 units of penicillin and 125 μg. streptomycin per ml. The suspension was clarified by centrifugation at 2500 rpm for 20 minutes, and the supernatant recentrifuged at 5000 rpm for 10 minutes. This supernatant was divided into aliquots and placed in a mechanical deep freeze at −60° C. When filtrates were prepared from spleen suspensions, antibiotics were omitted from the suspending medium, centrifugation was carried out as described, and the material was filtered through a Selas 03 candle. The filter was checked for ability to retain bacteria by adding *Serratia marcescens* to the suspension prior to filtration and culturing the material before and after filtration.

Control spleen suspensions and filtrates were made in the same manner as with infected spleens. Also, a serial intravenous passage line beginning with normal "General Purpose" mouse spleen was carried out simultaneously with the virus passage line; six serial passages were made, and in no instance did the passage animals develop splenomegaly.

Plan of experiments.—Serial tenfold dilutions of infected spleen suspension were prepared in chilled physiologic saline solution; the dilution tubes were held in an ice bath, and the materials were inoculated within an hour after preparation. All inoculations were made intravenously, since comparative experiments demonstrated that this route of inoculation produced more rapid spleen enlargement than intraperitoneal inoculation. The inoculum size was 0.2 ml. per mouse. Five or 10 mice were inoculated per dilution for each intended time of harvesting, and the mice to be killed at various times were designated at the beginning of the experiment. Two types of controls were used: uninoculated mice and/or mice inoculated with 10 percent suspension of normal "General Purpose" mouse spleen prepared in the same manner as the virus suspension.

At the designated time, mice were killed by cervical fracture, and the spleens removed and weighed individually on a Shadowgraph tumor-weighing scale, Model #4142.[5] Weights were read to the nearest 5 mg.

Mice that were obviously stunted in growth (body weight of less than 15 gm. at the time of death, when most mice weighed 18 to 22 gm.) were excluded from the experiment. It has been observed repeatedly that stunted mice did not develop splenomegaly comparable to that of their cage mates; since the occasional stunted mice did not fall in a particular dose range, their exclusion was not expected to bias the results. Mice that died of the disease were also excluded, except those that died on the day designated for killing. Death of animals was not a significant problem in the routine 2-week test, since few deaths occurred with preparations of the usual potency, and with highly potent preparations the deaths were in the lowest dilutions, which were outside the linear dose-response range used for analysis.

To confirm the observation that the splenomegaly was a manifestation of the leukemia, occasional impression smears were made of enlarged spleens. These were stained with Wright's stain and examined for the large mononuclear cells characteristic of the disease.

EXPERIMENTS

Dose-Response Relationship at Various Times After Inoculation

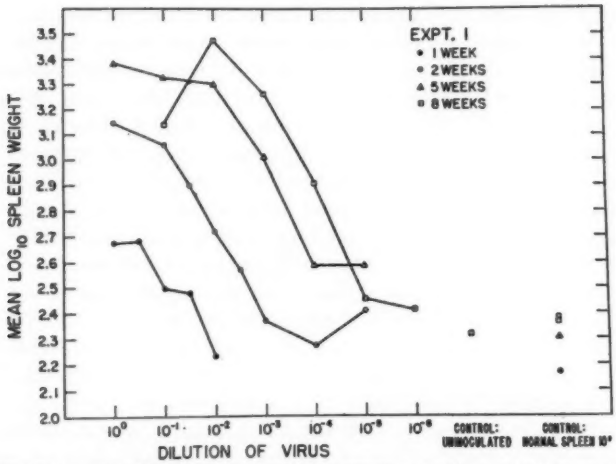
Text-figure 1 presents results of an initial experiment in which groups of mice inoculated with various dilutions of virus were killed at different times after inoculation; mean log spleen weight of 5 mice per point is plotted against dilution of virus inoculated. After the 1st week, the curves tended to be S-shaped, with a lower plateau in the range of control spleen weights at higher dilutions of virus, an approximately linear region, and an upper shoulder when mean log spleen weight exceeded $10^{3.0}$ to $10^{3.2}$ mg. Much variability of spleen weight was often seen in the upper shoulder region, primarily due to the hemorrhage and necrosis frequently seen in greatly enlarged spleens.

The existence of a region of linearity in the dose-response function, over a distance equivalent to approximately 2 $\log_{10}$ units of virus dose, indicated that this function could be used to quantitate the agent. The data described in text-figure 1 also indicate that the time when a certain spleen size is attained is a function of dose; thus it would be feasible to base a rough assay on the time when the spleen attains palpable size, that is, at about 500 mg., without the need for killing the mice.

Regression Analysis of 2-Week Dose-Response Functions

Since the 2-week period provided a degree of sensitivity adequate for many purposes in a relatively short period, 2-week assays were selected

[5] Exact Weight Scale Co., Columbus, Ohio.



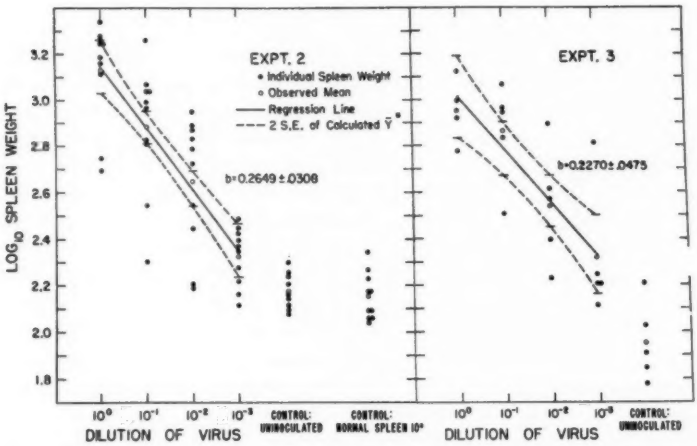
TEXT-FIGURE 1.—Relation of spleen-weight response to dose of virus as determined at 4 intervals after inoculation.

for further study and were subsequently adopted as a routine procedure for virus quantitation.

Because of the plateau regions at both extremities of the linear portion of the curve, calculation of regression lines was confined to the region of the curve between the highest dilution of virus-producing mean log spleen weight of 3.0 or greater and the lowest dilution with a mean within 0.15 log units of the simultaneous control value. For example, for the 2-week curve in text-figure 1, the region of virus dose 10^{-1} to 10^{-3} met these criteria. Regression lines were fitted by the method of least squares (*10*), and standard statistical procedures for regression analysis were applied (*10, 11*).

Text-figure 2 shows dose-response curves for 2 representative experiments with the observed individual spleen weights and means, the calculated regression line, and the 95 percent confidence limits (2 standard errors on each side of the regression line) of the calculated mean log spleen weights at each dose level. As seen in text-figure 2, a point at a vertical distance of 2 standard errors from the calculated regression line was at a horizontal distance from that line equivalent to a difference in virus dilution of $10^{0.3}$ to $10^{0.7}$. This indicated that differences of this order of magnitude in potency of 2 virus preparations could be detected when the regression line of one and a single intermediate mean response of the other are known. When the regression lines of both preparations are established, the accuracy of the comparison is greater.

The value of a dose-response assay depends in large part on the steepness and reproducibility of the regression line; a steep line permits more precise quantitation, but where the range of responses is limited, as in the Friend virus assays, too steep a slope greatly limits the useful range of the test. The reproducibility of the slope is of particular importance



TEXT-FIGURE 2.—Regression analysis of 2 representative 2-week dose-response assays. *Indicates 2 standard errors of the calculated *mean* spleen weight on each side of the regression line; this does not refer to the standard error of the individual spleen weights.

when it is desired to make estimates of titer of a preparation on the basis of the response at a single dose level.

Table 1 presents the distribution of the calculated slopes of the regression lines for 47 consecutive dose-response assays with the 2-week endpoint; 7 assays are included in which the slope was based on responses at only 2 dose levels, generally because of exclusion of points on the upper shoulder. Forty-three (91%) of the 47 slopes fell in the range of 0.1560 to 0.3942; the standard errors of the slopes were generally of the order of one third to one fifth of the slope. Two of the assays recorded in table 1 had slopes of less than 0.05; both instances were apparently upper shoulder regions, the mean log spleen weights being between 2.9 and 3.0 for all 3 dose levels tested.

TABLE 1.—Distribution of slope of regression line in 47 consecutive dose-response assays

Slope (b) (*decline in mean log spleen weight per tenfold dilution of virus*)	Number of assays with slope in each range			Mean standard error of the slope (σ_b)
	Assays with 3 or more points	Assays with 2 points	Total	
0–0.099	2		2	0.068
0.100–0.199	4		4	0.054
0.200–0.299	25	2	27	0.052
0.300–0.399	8	4	12	0.074
0.400–0.499			0	
0.500–0.599	1	1	2	0.129
Mean number of mice per assay	17.8	12.6	17.1	

Calculation of Virus Titer

For expression of potency of virus preparations as determined by the dose-response assay, titer was arbitrarily defined as the dilution of the virus preparation, which corresponded to a mean spleen weight of $10^{2.7}$ mg. (500 mg.) on the regression line, *i.e.*, the dilution of the preparation calculated to produce spleen enlargement to 500 mg. in 2 weeks when inoculated intravenously in 0.2-ml. volume. This titer is referred to as the 500-mg. spleen-enlarging dose (SED500). The point 2.7 was selected since it was approximately at the midpoint of the linear portion of the curves and consequently least likely to be on a shoulder or to be unduly influenced by variations in slope of the regression line. This method of expressing potency has the advantage of being stated in terms comparable to those used in other virus assay methods. Table 2 records the computation of the regression line and titer in a representative assay (expt. 2 in text-fig. 2).

Reproducibility of Titration Results

Table 3 presents data on the reproducibility of titration results. Pooled mouse-spleen suspensions were prepared at the fourteenth and 26th serial passage level, distributed into small aliquots in screw-capped vials, and stored in a mechanical deepfreeze at −60° C. A fresh vial was removed for each test. The tests on pool #197 are divided into 2 groups, because between the tests on May 27, 1958, and July 9, 1958, the vials were inadvertently thawed and refrozen. Within the 2 groups of tests on pool #197, titers fell within 0.5 to 0.6 log of one another. In the tests on pool #426, the initial tests made on freshly prepared spleen suspension gave higher titers than those on the material after storage for 3 to 8 weeks, but the 4 tests on the stored suspension repeated within 0.5 log. The table also shows the 50 percent infectious dose (ID50) titers (*12*) calculated from the same data, with individual spleen weight of 500 mg. or greater as the criterion of infection; titers expressed in this manner agreed closely with those obtained from the regression analysis. However, in a number of instances, a definite ID50 titer could not be calculated, since the titration endpoint was not bracketed.

DISCUSSION

The 2-week dose-response curves obtained with the Friend virus provide a means for an accurate comparison of potencies of different preparations, but obviously show much lower titers than those obtained by prolonged observation for splenomegaly or death of mice receiving high dilutions of virus. However, for most quantitative work, what is required is a comparison of different potencies or titers rather than the "absolute"

TABLE 2.—Sample calculation of regression line and titer—data of experiment 2 (text-fig. 2)

(1) Log_{10} of dilution of virus (X_i)	(2) Number of mice per dose (n_i)	(3) Sum of individual $\log_{10}$ spleen weights in each dose group (ΣY_{ii})	(4) (1) × (2) (n_iX_i)	(5) (1) × (4) ($n_iX^2_i$)	(6) (1) × (3) (ΣX_iY_{ii})
0	10	31. 2878	0	0	0
−1	10	28. 8451	−10	10	−28. 8451
−2	10	26. 4783	−20	40	−52. 9566
−3	10	23. 2475	−30	90	−69. 7425
Total	40 (N)	109. 8587 ($\Sigma\Sigma Y_{ii}$) = "C"	−60 (Σn_iX_i) = "D"	140 ($\Sigma n_iX_i^2$) = "E"	−151. 5442 ($\Sigma\Sigma X_iY_{ii}$) = "F"

$$\text{Slope } (b) = \frac{F - \frac{C \times D}{N}}{E - \frac{D^2}{N}} = \frac{-151.5442 - \frac{(109.8587)\ (-60)}{40}}{140 - \frac{(-60)^2}{40}} = 0.2649$$

$$Y \text{ intercept } (a) \quad (a = \overline{Y} - b\overline{X}) = \frac{C}{N} - b\frac{D}{N} = \frac{109.8587}{40} - 0.2649 \times \frac{-60}{40} = 3.1439$$

$$\text{SED500} = \text{Log}_{10} \text{ titer}_{(Y=2.7)} = \frac{a-2.7}{b} = \frac{3.1439-2.7}{0.2649} = 1.68$$

TABLE 3.—Reproducibility of assays on aliquots of virus suspension

Pool No.	Date of assay	Dilution range analyzed	Number of mice	$b \pm \sigma_b$*	Titer†	
					SED500	ID50
197	January 31, 1958	$10^{0} - 10^{-3}$	24	.3393 ± .0404	2.05	1.92
January 29, 1958	April 4, 1958	$10^{0} - 10^{-3}$	40	.2649 ± .0308	1.68	1.89
14th passage	May 27, 1958	$10^{-1} - 10^{-3}$	19	.3697 ± .0984	1.95	≥2.00
	May 27, 1958	$10^{0} - 10^{-3}$	29	.2115 ± .0491	2.25	≥2.29
197 F‡	July 9, 1958	$10^{-1} - 10^{-3}$	20	.2786 ± .0906	0.99	≤1.10
January 29, 1958	August 19, 1958	$10^{0} - 10^{-3}$	18	.2788 ± .1010	0.52	≤0.75
	October 16, 1958	$10^{0} - 10^{-3}$	15	.2618 ± .0639	0.63	0.83
	November 14, 1958	$10^{-2} - 10^{-4}$	13	.1560 ± .0838	4.15	≥4.38
	November 14, 1958	$10^{-2} - 10^{-4}$	15	.1658 ± .0469	4.79	≥4.50
426						
November 14, 1958	December 5, 1958	$10^{-2} - 10^{-5}$	19	.2478 ± .0292	3.13	3.40
26th passage	January 7, 1959	$10^{-2} - 10^{-4}$	15	.2233 ± .0394	3.27	3.17
	January 14, 1959	$10^{-2} - 10^{-4}$	14	.2074 ± .0352	3.59	3.52
	January 16, 1959	$10^{-2} - 10^{-4}$	15	.2138 ± .0425	3.55	3.50

* Slope ± standard error of slope.

† $\log_{10}$ of the number of infectious doses per 0.2 ml.

‡ Same as pool 197, but subjected to an additional thawing and freezing.

infectivity endpoints obtainable in the most sensitive assay system. In fact, a short-term assay giving a relative indication of titer is often necessary for accurate results, since prolonged assays provide more opportunity for action of extraneous variables.

It is necessary to keep in mind the possibility that different passage lines of virus may differ in "potency," *i.e.*, the rapidity with which a given number of infectious doses produces splenomegaly. If such should occur, 2-week assays would not bear a constant relation to the actual titer determined by prolonged observation. For this reason, relative titrations are most meaningful for comparison of titers of portions of the same virus preparation after application of various procedures, or for comparison of virus preparations relatively close in passage history.

Although noninbred Swiss mice are generally considered inferior to inbred mice for tumor studies, in the Friend virus assays they appear to represent the optimal system. Friend (*8*) found only Swiss and DBA mice, in a series of mouse strains, to be susceptible to the agent. In our experience, inbred DBA mice have been somewhat less susceptible than Swiss, and the reproducibility of response has not been greater. Although we have found (*13*) that high-titered preparations induce the disease in other inbred mouse strains, including AKR/LwN and C3H/P, these strains are far too insensitive for use in quantitation. The intravenous route of inoculation is necessary for a sensitive assay procedure. When a standard virus preparation was titrated simultaneously by the intraperitoneal and intravenous routes, the slopes of the dose-response lines were identical, but the SED500 in the intraperitoneally inoculated group was 1.4 $\log_{10}$ units lower than in the intravenous group.

The graded-response assay described here has several advantages over standard extinction dilution titrations. A death endpoint requires several months for obtaining results and is too erratic for a short observation, relative titration. Fifty percent infectious endpoints based on spleen weight of greater than 500 mg. at 2 weeks as the criterion of infection provide an acceptable method of calculating titers, but are considered inferior to dose-response assays in several respects. (1) Dose-response assays often permit determination of titer, when the endpoint is not reached, by extrapolation of the regression line; this permits use of fewer animals per titration, and results in salvaging of titrations. (2) The dose-response assay provides an indication of titer from data obtained at a single virus dilution. (3) The character of the dose-response curve provides a check on the normality of the mouse response, and thus may well detect influence on spleen response of extraneous microbial agents.

It is well recognized that spleen weight varies markedly with changes in physiologic status of the mouse; food deprivation, infection, and stress may produce rapid and marked alteration in spleen weight. It is therefore necessary in the procedure described here that special attention be given to the care and condition of test animals. Incorporation of a broad-spectrum antibiotic in the inoculum or drinking water may be advisable to prevent bacterial and *Eperythrozoon* infections.

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Embryology of Testicular Teratomas in Strain 129 Mice [1,2]

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SUMMARY

Based on observations of teratomas in strain 129 infant mice, the following sequence of development is proposed: The most primitive tumors are composed of clusters of embryonal cells with germ cells interspersed. Some of these cells form epithelial vesicles surrounding pools of blood and cellular debris. Approximately half these vesicles are morphologically different from the other half, signifying the formation of layers of cells homologous to ectoderm and endoderm. Mesoderm surrounds the vesicles. Ectoderm is transformed into neuroepithelium; teratomatous endoderm gives rise to alimentary and respiratory epithelium; and mesoderm becomes muscle and cartilage. Embryoid bodies with inverted germ layers are observed in enormous numbers in ascitic fluid resulting from intraperitoneal grafts of transplantable teratomas. The endoderm in these formations resembles that in 6-day-old mouse embryos. The endoderm of the early primary tumors is similar to alimentary epithelium in 9-day-old embryos. Apparently the environment, whether fluid or cellular, exerts an important influence on the configuration of endodermal cells. The arrangement of endodermal cells in embryoid bodies indicates that they have centrifugal migration tendencies, which may explain, tentatively, the inversion of the germ layers in mouse embryos. In most of the early teratomas there is direct continuity between the germinal epithelium and teratomatous elements. This suggests, but is not critical proof, that testicular teratomas originate within the tubules of the fetal mouse testis.—J. Nat. Cancer Inst. 23: 1249–1295, 1959.

TESTICULAR TERATOMAS develop spontaneously in approximately 1 percent of strain 129 male mice (*1*, *2*). They are congenital tumors with the capacity to produce many types of tissues, such as several kinds of epithelia, nervous tissue, cartilage, bone, and marrow; skeletal, cardiac, and smooth muscle, fat, and embryonic mesenchyme. There is no increase in the frequency of these tumors with advancing age, indicating

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that they are initiated prenatally during a critical period, and not later. The relatively high incidence of these teratomas, which are initiated during a given stage of development, has made it possible to study them from early through later stages.

The most primitive tumors we have examined, in newborn mice, are composed of clusters of embryonal cells with germ cells interspersed. In slightly more advanced tumors, two morphologically different types of epithelia appear, and it will be shown that they are homologous with the primary germ layers, ectoderm and endoderm. Evidence, but not final proof, will be presented that the cell of origin of these tumors is a component of the seminiferous tubules, most likely the germ cell.

MATERIALS AND METHODS

The primary testicular teratomas were derived from several colonies of strain 129 mice maintained at the Jackson Memorial Laboratory. A few tumors were found in litters from mothers treated, while pregnant, with various agents. However, there was no indication that any experimental treatment initiated or promoted teratocarcinogenesis of the testis.

To obtain tumors from mice of known ages, pens with pregnant females were observed daily, except Sunday, for new litters. Mice were classified as 0 days of age when found in animals autopsied on the day of birth. Actually some of these animals may have been as old as 24 hours. Males were overanesthetized with chloroform, the abdominal contents exposed, and the testes of infants observed with a stereoscopic microscope of 10× magnification. All teratomas and testes suspected of being tumorous were fixed in Vandegrift's solution, serially sectioned at 6 or 8 mμ and stained with Mayer's acid hematoxylin followed by 0.3 percent eosin in alcohol.

The testicular teratomas of strain 129 mice 7 days of age and older are readily identified during *post-mortem* examination, and some may be detected in animals as young as 4 days. The tumors enlarge the testes, and their homogeneous structure contrasts with the adjacent testicular tubules. Teratomas are always accompanied by hemorrhage which facilitates their identification. However, the testes of young (0-to-5 days) strain 129 mice frequently contained localized areas of extravasated blood, and it was necessary to study many serial sections of testes suspected of being tumorous, in 0- to 4-day-old animals, to find one that actually had a teratoma.

The derivation of the transplantable teratomas and the procedures used to maintain them have been described previously (*3*). Some grafts were homogenized in saline with a glass homogenizer, and 0.5 ml. of the resulting suspension injected intraperitoneally. These intraperitoneal grafts were serially transplanted by inoculating 0.2 ml. of ascitic fluid containing single cells and groups of cells.

OBSERVATIONS

A summary of some characteristics of the tumors in mice 0 to 4 days old is presented in table 1. In addition to those included in table 1, the observations presented here are based on tumors from four 5-day-old, four 6-day-old, and forty 11- to 14-day-old mice. Of a total of 93 teratomas in animals less than 20 days of age, 59 were in the left testis, and 31 were in the right testis. There were 3 teratomas in which laterality was unknown.

TABLE 1.—Some characteristics of early testicular teratomas in strain 129 mice 0 to 4 days old

Designation	Age of host (*days*)	Laterality	Localized hemorrhagic areas	Continuity of seminiferous epithelium and tumor	Ectopic germ cells	Number of foci
LS 1250	0	L	Present	No	?	1
LS 1448	0	?	Present	?	Yes	1
LS 1517	0	R	Present	?	Yes	1
LS 1571	0	R	Present	Yes	Yes	2
LS 1581	0	?	Present	No	Yes	1
LS 1956	1	?	Present	No	Yes	1
LS 1119	2	L	Present	Yes	Yes	2
LS 1293	2	L	Present	Yes	Yes	3
LS 1131	3	R	Present	No?	Yes	1
LS 1153	3	L	Present	Yes	Yes	1
LS 2058	3	R	Present	Yes	Yes	1
LS 1233	4	L	Present	Probably	*	†
LS 1886	4	L	Present	Yes		
LS 1945	4	L	Present	Yes		
LS 2032	4	L	Present	Yes		
LS 2042	4	L	Present	Yes		

*Ectopic germ cells are not readily distinguished from embryonal cells in animals 4 days of age.

†The tumors in animals 4 days of age occupy a greater proportion of the testis than in younger animals, which makes it difficult to distinguish single foci from growths resulting from fusion of several foci.

Composition of Early Primary Teratomas

The teratomas obtained from animals of any given age showed variation in degree of complexity and in developmental maturity. For instance, one precocious teratoma found in the testis of a 4-day-old mouse contained recognizable neuroepithelium, cartilage, and columnar epithelium, while others from animals of the same age were less mature, composed of mesenchymal cells and primitive epithelia. It is possible that some tumors were initiated earlier than others, with the result that tumors in animals of the same age showed variations in degree of maturity. Development proceeds rapidly during early embryonal stages, and slight differences in onset or rate of tissue differentiation may be reflected by conspicuous differences in degree of maturation. It is emphasized, however, that there is a tendency toward increasing complexity and maturity in progressively older tumors.

The teratomas will be described in the order of increasing age of the mice in which they were found.

Zero day.—The microscopic tumors in the testes of mice autopsied on the day of birth are comparatively simple. The most simple is extratubular, composed of a cluster of morphologically undifferentiated embryonal cells with apparently normal germ cells interspersed (figs. 1 and 17). There is no indication of epithelial formation, nor is there any apparent continuity between neoplastic elements and adjacent seminiferous tubules. In another tumor there are two separate teratomatous foci. One of these foci, similar to the simple tumor mentioned, is composed of embryonal cells with germ cells interspersed but with incipient epithelium formation (figs. 2 and 18).

The other tumors of newborn mice are more advanced in development (figs. 3, 4, 19, 20, and 21). The first indication of organization is the alignment of cells in epithelial arrangements around extravasated blood and cellular debris (figs. 19 and 20).

In slightly more advanced tumors of newborns, 2 morphologically different types of epithelia are recognized. One is composed of cells which become elongated with their long axes oriented toward the center of the pool of blood. This epithelium is arranged in an orderly manner with well-defined internal and external limiting membranes. The component cells have oval, distinctly outlined nuclei with finely granulated chromatin and 2 or 3 well-defined nucleoli. The cytoplasm is slightly basophilic, sharply outlined by the cell membranes. As may be seen in figure 21, this epithelium resembles the ectodermal epithelium in mouse embryos 6 days *post coitum* (fig. 59). Evidence will be presented that this epithelium is homologous with embryonic ectoderm, and that ectodermal derivatives, usually neuroepithelium, but occasionally skin, develop from it.

The second morphological variety of epithelium (figs. 21, and 24 to 30) develops concurrently with the ectodermal epithelium described, and also forms irregular spheres enclosing pools of blood and cellular debris. The cellular outlines are less distinct than in the ectodermal epithelium, the cytoplasm slightly eosinophilic and finely granular. The nuclear membranes are well defined. This epithelium is identified as endoderm, since it gives rise to endodermal, but not ectodermal, derivatives. In newborns, the teratomatous endoderm lacks an internal limiting membrane, and presents an irregular appearance.

The endodermal epithelium of early primary teratomas resembles pharyngeal epithelium of 9-day-old mouse embryos (fig. 35) rather than the endoderm of 6-day-old embryos (fig. 59).

In addition to ectoderm and endoderm, mesoderm is represented by mesenchymal cells situated between them. The 2 epithelial types are actually different from one another and represent distinct entities: (1) They are found regularly as primary structural arrangements in early tumors. (2) Both types may be seen forming *de novo* in older primary teratomas and in some teratomas that have been serially transplanted for over 5 years. (3) There is rarely, if ever, an admixture of the 2 types of epithelia; either ectoderm or endoderm forms the entire lining of vesicles. (4) A blue-staining substance is commonly seen in endodermal, but not

in ectodermal, vesicles in teratomas of mice a few days old. (5) Developmental stages may be traced from ectodermal epithelium to neuroepithelium, the most commonly found component of the older teratomas, and from endodermal epithelium to alimentary and respiratory epithelium, with satellite glands and layers of encircling smooth muscle.

In summary, the most simple testicular teratomas of newborns are composed of accumulations of embryonal cells which selectively sort themselves out and become arranged around pools of blood and products of cellular deterioration as epithelial vesicles resembling either embryonic ectoderm or endoderm. The tumors of newborns are small, but contain many mitotic figures, indicating rapid growth. These early tumors are usually associated with ectopic germ cells, and frequently are directly continuous with seminiferous epithelium (figs. 53 to 58).

One to 4 days.—The tumors of this age group are found in hemorrhagic areas and are larger than those of newborn mice. They are composed of ectodermal and endodermal epithelial vesicles and embryonic mesenchyme representing mesoderm (figs. 5 to 13, and 22 to 30). In some areas the ectoderm appears to be proliferating (figs. 22 and 26) presenting a picture similar to mesoderm formation in the mouse embryo at the primitive streak stage. The transformation of ectodermal epithelium in the tumors of 3-day-old mice into immature neuroepithelium is the first striking histogenetic event following germ-layer formation. However, there are transitional stages between primary ectoderm and neuroepithelium, and the onset of neuralization is difficult to pinpoint (fig. 31). Nuclei become displaced away from the lumina of the vesicles (fig. 32), and the epithelium resembles the thickened stratified neuroepithelium lining the neural groove of 9-day-old mouse embryos (fig. 36). In both there are numerous mitoses in the ependymal layer of cells.

The nonepithelial embryonal cells also show many mitotic figures and may form new vesicles, ectodermal or endodermal, by cavitation. At 3 days the endodermal, but not the ectodermal, vesicles frequently contain a substance which stains light blue with hematoxylin. This substance is also present in older primary and transplantable teratomas in vesicles lined by primitive endoderm.

In 4-day-old animals, the teratomatous endoderm becomes more organized, with the appearance of an external limiting membrane, and the cells lose their rough, irregular outline and become cuboidal or columnar (fig. 27).

Five to 8 days.—From 5 to 8 days, the teratomas grow considerably and usually enlarge the gonad (figs. 14 to 16). New ecto-, endo-, and mesodermal elements are formed, while the previously established layers become more completely differentiated. Some tumors in animals of this age group are like the more advanced ones in the earlier age group. They contain ectoderm, endoderm, and mesoderm and, in addition, neural vesicles with marginal layers, precartilaginous mesenchyme, and myoblasts (figs. 42 to 44, and 49). Others, more primitive, have less mature neural vesicles and lack identifiable precartilaginous mesenchyme. Fetal

heart muscle is seen in one 6-day-old animal (fig. 51). Further development of central nervous tissue in tumors of 6-day-old animals is indicated by fascicles of nerve fibers (fig. 34).

Eight to 9 days.—Pseudostratified columnar ciliated epithelium is observed at 8 and 9 days, as well as structures similar to embryonal neural canals, neuroglial tissue, and nerve cell bodies (figs. 33, 38, and 39). Usually there are condensations of precartilaginous mesenchyme and myoblasts.

Ten to 11 days.—At 10 and 11 days of age, ciliated epithelium (fig. 40), goblet cells (fig. 41), islets of cartilage with peripheral ossification (fig. 47), myoblasts (fig. 50), and, occasionally, striated muscle are seen. Irregularly shaped neural tubes are observed, with ependymal, mantle, and marginal layers and with nerve fibers emitting. Notochord with vacuolated cells is seen (figs. 45 and 46). The primordia of most tissues found in older teratomas are recognizable in tumors of animals 10 days of age.

Eleven to 19 days.—By 17 to 19 days, most of the teratomas contain bone marrow (fig. 48), striated muscle, glandular structures (fig. 52), and stratified squamous epithelium. The sequence of tissue differentiation in the teratomas of strain 129 mice is similar to that in normal mouse development.

The composition of teratomas of animals 20 to 30 days of age has been described in detail in an earlier paper (*2*). The proportion of tumors containing embryonal and immature tissues decreases with advancing age of the host.

Continuity of Seminiferous Epithelium and Neoplastic Elements

As noted in table 1, there is direct continuity between the seminiferous epithelium and neoplastic elements in many early tumors (figs. 53 to 58). In several tumors, such continuity was observed in more than one place. Typically, the diameter of the tubules is increased by embryonal cells and early neuroepithelium (figs. 56 to 58). In one case, a small tumor was observed within a testicular tubule adjacent to a large primary teratoma. Whether the intertubular mass is a separate focus or whether it represents invasion of the tubule by the adjacent tumor is impossible to determine. since the basement membrane cannot be resolved in one area, and it is possible that it is not intact.

Embryoid Bodies in Metastatic Growth and in Transplantable Teratomas of Strain 129 Mice

Even though the primary germ layers are routinely noted in early teratomas, they do not assume the spatial relationships found in early normal mouse embryos. However, under certain conditions, the teratomas of strain 129 mice produce thousands of formations similar to early mouse embryos (fig. 59). These have been observed in a metastatic growth of a primary tumor (fig. 61) and in the ascitic fluid resulting from

intraperitoneal grafts of some sublines of transplantable teratomas (figs. 62 and 63). Occasionally they are seen in subcutaneous grafts (figs. 60 and 64). Clusters of embryonal cells are enclosed by a single-layered epithelium that resembles embryonic endoderm. The epithelial cells are cuboidal or squamous, with round or ellipsoidal nuclei; the cytoplasm is eosinophilic. Frequently the enclosed embryonal cells are epithelial and resemble early mouse ectoderm. These free-floating bodies enlarge considerably and become spherical cysts. The endodermal epithelium becomes transformed into hematopoietic yolk sac, and the ectoderm may become neuroepithelium. Occasionally, muscular movements are observed.

In these embryoid bodies the endoderm takes an external, the ectoderm an internal, and the mesoderm an intermediate position (*3*), as in normal mouse embryos of approximately 7 days of age. While the endoderm in the early *primary* tumors resembles early pharyngeal epithelium of normal mouse embryos, the endoderm of the embryoid bodies in the metastatic and intraperitoneal growths is like the proximal and distal (yolk sac) endoderm in 6- to 7-day-old mouse embryos.

DISCUSSION

Based on the observations on the early testicular teratomas of strain 129 mice presented here, the following sequence of postnatal development is proposed: The most primitive tumors are composed of clusters of morphologically undifferentiated cells with many mitotic figures. Certain of these cells make contact, adhere to one another, and assume epithelial arrangements surrounding pools of extravasated blood and products of cellular necrosis. Approximately half these epithelial vesicles become morphologically different from the other half, which signifies the formation of the two primary germ layers, ectoderm and endoderm. The vesicles are either ectodermal or endodermal, rarely, if ever, mixed. Embryonal and mesenchymal cells surround and separate the ecto- and endodermal vesicles from one another. After this sorting out of cell types, morphological differentiation of ectoderm into primarily neuroepithelium, endodermal into alimentary and respiratory epithelia, and mesodermal into cartilage and muscle derivatives occurs.

The integration of cells of early teratomas into histogenetic complexes is reminiscent of experimental developmental systems involving cell movements, selective adhesion of like cells, and subsequent differentiation. Experimental embryologists have used extensively methods of dissociating embryonic tissues and reaggregating the component cells to study factors involved in organogenesis and embryogenesis. They have demonstrated that directed cell movements and affinities and disaffinities between cells arise as corollaries of differentiation (*4–10*). Holtfreter (*5*) pointed out that complex patterns of organization may arise not only from dis-

sociated previous patterns, but may be created *de novo* in an initially homogeneous mass of cells.

Townes and Holtfreter (6) dissociated amphibian medullary plate and allowed the component cells to reaggregate. They found that the central lumen, which normally results from the infolding of the plate, can be formed equally well by way of secondary cavitation. The ependyma-lined neural vesicles in the mouse teratomas likewise are formed by cavitation, and not by the formation and closure of neural folds.

The description of developmental stages of testicular teratomas in strain 129 mice and, in particular, the observations on embryoid bodies suggest that cells selectively sort themselves out into germ layers within a morphologically homogeneous mass of cells, as has been shown for other vertebrates which have been experimentally investigated.

After the formation of the germ layers in the early teratomas, embryonic neuroepithelium is the first recognizable tissue to appear. Holtfreter (*5*) has shown that a wide variety of tissues, which are noninductive when alive, acquire the faculty of inducing neural tissue when they are killed by any of several physical or chemical means. He postulated that it was simply the toxicity of the killed materials which produced the inductive activity. A characteristic feature of the early teratomas described here is that they are regularly accompanied by cytolyzed material and hemorrhage into the testis. There may be a causal relationship between dying cells and neuralization of the adjacent pluripotent cells in the early mouse teratomas, as there seems to be in Holtfreter's amphibian embryonic material. However, in the tumors of older animals and in the transplantable tumors, neuralization occurs in the absence of hemorrhage and deteriorating cells.

Falin (*11*), Bagg (*12*), and Carleton *et al.* (*13*) suggested that necrosis was important in the genesis of zinc-induced testicular teratomas of the fowl. Cellular degeneration was observed in all teratomas of strain 129 infant mice, suggesting the possibility that cytolytic influences may be a factor in the genesis of these tumors.

The germ layers are inverted in the embryoid bodies formed in the ascitic fluid of mice with intraperitoneal grafts. The endoderm takes an external, the ectoderm an internal, and the mesoderm an intermediate position, as in normal 6-day-old mouse embryos. The teratomatous endoderm of the embryoid bodies resembles the proximal endoderm of early mouse embryos, while the germ layers are still inverted (figs. 59 to 64). In the embryoid bodies and in normal 6-day-old embryos, the endoderm surrounds other embryonal cells and is exposed to a fluid medium. The endoderm in the early *primary* tumors, on the other hand, lines lumina and resembles pharyngeal epithelium of 9-day-old mouse embryos (figs. 35 and 37). Nearly all the endoderm in teratomas serially transplanted subcutaneously for over 5 years is of the alimentary or respiratory type and exhibits the structure characteristic of lining endoderm. However, when these tumors are homogenized and placed in a fluid medium (ascitic), the endodermal cells that form the outer layer of em-

bryoid bodies assume a configuration similar to the endodermal cells of early mouse embryos.

Embryoid bodies with endoderm of the type seen in 6-day-old mouse embryos have never been observed in primary teratomas of strain 129 mice of any age. They were observed in a retroperitoneal metastatic growth (fig. 61) and occasionally they have been seen in transplantable teratomas grafted subcutaneously (figs. 60 and 64), but they are extremely rare in the solid growths compared with the enormous numbers seen in the ascitic fluid. Embryoid bodies in the solid tumors are surrounded by small pockets of fluid, such as those formed by the yolk sacs of normal embryos.

Preliminary results indicate that when small groups of embryonal cells of teratomatous origin are cultivated *in vitro*, endoderm encloses other embryonal cells and resembles the endoderm of 6-day-old normal embryos. Apparently the environment exerts an important influence on the position and morphology of endodermal cells. It is the exposure to the external fluid environment rather than an influence emitted by the underlying embryonal cells that determines the shape of the endodermal cells, since cysts composed solely of endoderm of the covering type seen in 6-day-old embryos, and devoid of ectoderm or mesoderm, have been observed in the ascitic fluid.

The arrangement of the endodermal cells in the embryoid bodies indicates that they have centrifugal migratory tendencies within small masses of cells, and that they have disaffinities to ectodermal cells. If further study confirms this, it would serve as an explanation for the inversion of the germ layers in mouse embryos.

Although several investigators have described embryoid bodies in human gonadal tumors (*14–22*), they are apparently rarely observed. Peyron (*14*), the first to report embryoid bodies in human testicular teratomas, found that they were particularly abundant in the spermatic veins of the affected gonad. As appears to be true of the mouse, a fluid environment apparently promotes the formation of embryoid bodies from human teratomatous embryonal cells. The infrequency of embryoid bodies in human testicular teratomas may be due to the fact that they are formed under particular conditions rather than to a lack of capacity of teratomatous cells to form these structures.

In this discussion, the terms ectoderm, endoderm, and mesoderm are used to indicate morphological entities as well as prospective potencies. We do not hesitate to designate the two types of epithelia, which develop in the early teratomas, as ectoderm and endoderm. The former invariably gives rise to ectodermal, and the latter to endodermal derivatives. Oppenheimer (*23*) discussed experiments, including those of Mangold, which showed that the germ layers were able to produce a greater variety of tissues than they do in normal development, and Trinkaus (*24*) has argued convincingly against the theory of irreversible differentiation of tissue cells. It is not implied that there are permanent irreversible physiological differences between the germ layers in teratomas or that

they might not possess a greater developmental versatility than they express under the conditions we have observed them.

Both Nicholson (*25*) and Willis (*26*) have argued against germ-layer formation in human testicular teratomas. Nicholson found no evidence of delamination of primary germ layers in teratomas, and suggested that their tissues undergo "apparent promiscuous and haphazard differentiation *in situ*." According to Willis, the three germ layers of normal development have no real relevance in the structure of teratomas, for they show no evidence whatever of orderly delamination. However, in a description of histogenesis of neural tissue in teratomas, Willis (*27*) emphasized that frequently simple epithelium, neuro-, and epidermal epithelium were in direct continuity with one another. He concluded that a simple plastic epithelium in teratomas has potencies of becoming either neuro- or epidermal epithelium, and that "This mode of origin of neural tissue resembles that of the growing embryo, in which also neural and epidermal elements arise by divergent differentiation from a simple plastic epithelium, the primary ectoderm."

The observations on the early testicular teratomas in strain 129 mice demonstrate conclusively that adult elements arise by differentiation of embryonal cells. Pierce and Dixon (*28*) came to the same conclusion based on their studies of subline 402A VI of a transplantable teratoma in strain 129 mice.

Usually the primary testicular teratomas in strain 129 mice cease to grow at the time they lose embryonal components. It is apparent that progressive growth of the tumors is due to embryonal cells. Jackson and Brues (*29*) studied an ovarian teratoma of the mouse and concluded that the most immature cells were responsible for the growth of the tumor. They postulated a "balancing process" which maintained (stem) cell division and maturation in their tumor at such relative rates that both embryonal and mature tissues persisted for a long time. We have maintained a highly pleiomorphic progressively growing transplantable teratoma (402A VI) for over 5 years, and its behavior supports this point of view. However, this "balancing process" may be lost, resulting in an embryonal cell tumor, or it may be altered in the direction of decreasing potencies. Occasionally grafts of subline 402A VI differentiate into adult-type tissues and lose the attribute of progressive growth, but most grafts are a composite mixture of several kinds of tissues, including immature and embryonal.

Pierce and Dixon (*28*) found cysts composed of yolk sac, undifferentiated cells, and mesenchyme in the ascitic fluid of mice with intraperitoneal grafts of a transplantable mouse testicular teratoma of strain 129 origin. They felt that ". . . the composition of the cysts suggests the possibility that they are fetiform derivatives of the teratocarcinoma, but at present insufficient evidence is available to prove the contention." When grafted subcutaneously, the cysts gave rise to full-blown, three germ-layered teratomas, which supports the hypothesis that adult-appearing tissues in a teratoma arise from undifferentiated and presumed multipotent cells. As

Pierce and Dixon point out, however, no one has proved this. We have observed formations such as those described by Pierce and Dixon, and in addition we have observed muscle and immature neural tissue in some of them. However, this does not detract from their tentative conclusions, since it is apparent that the differentiated elements in larger cysts arise from undifferentiated elements observed in smaller ones.

In this discussion, all the primary tumors of the testes in strain 129 mice and the transplantable tumors derived from them are designated teratomas. According to the classification of testicular tumors proposed by Friedman and Moore (*15*), the earliest tumors would be termed "embryonal carcinoma" (composed of undifferentiated cells), those in mice 5 days and older, "teratocarcinoma" (pleiomorphic tumors in which both differentiated teratoid structures and histologically malignant elements are present), and those in most animals over 1 month of age, "teratoma" (composed of mature tissues). However, as explained previously (*2*), we prefer to use the term "teratoma," according to the classification of Willis (*30*), since most tumors become completely differentiated into adult-type tissues and do not grow progressively.

Melicow (*31*) discussed the relationships between germinal tumors of the human testis and concluded that the three tumors, designated embryonal carcinoma, teratocarcinoma, and adult teratoma, might represent consecutive stages in the development of a single kind of neoplasm. Teratocarcinoma might be considered a partially differentiated embryonal carcinoma, and might be regarded as an intermediate stage between embryonal carcinoma and adult teratoma in which complete differentiation of embryonal elements has taken place. According to Melicow, embryomatous tumors of the testis start out as anaplastic primitive embryonal cells and tend to progress toward differentiation. Friedman and Moore (*15*) also expressed the opinion that adult teratomas represent matured teratocarcinoma. These points of view receive strong support from our observations.

Foulds (*32*) defined tumor progression as ". . . the development of a tumor by way of permanent, irreversible qualitative change in one or more of its characters." The primary testicular teratomas in strain 129 mice progress in an unusually dramatic fashion, changing irreversibly and rapidly from embryonic to adult-like growths. Six sublines of a transplantable teratoma, derived from a single primary tumor (402), have been shown to evolve into different histogenetic categories (*3*). One category is represented by transplantable tumors which, like most of the primary growths, differentiate completely into adult tissues, and lose their embryonal components and the capability of progressive growth. Tumors in other categories invariably grow progressively, but during the course of many transplant generations have become restricted in embryological competence. The evolution of histogenetic differences has been offered as an example of tumor progression as judged by morphological characteristics.

In most of the early teratomas described here, there was direct con-

tinuity between the germinal epithelium of the testicular tubule, teratomatous neuroepithelium, and primitive embryonal epithelium. In these cases, the testicular tubules were usually enlarged by the presence of dividing neoplastic cells, and the basement membranes were disrupted, giving the appearance that the teratomas had originated within the tubules and that pressure due to growth had ruptured the seminiferous epithelium and basement membrane, spilling out tumor cells. The possibility remains, however, that the tumors had originated in the interstitial spaces and infiltrated into the lumina of the tubules. Teratomatous elements within seminiferous tubules have been reported by Peyron (*14*) and Melicow (*31*), in the human being, and by Falin (*11*), in the fowl. Germ cells were observed interspersed among early tumors in the mouse (fig. 17), even when the tubules had not been apparently disturbed. Extratubular germ cells in newborn strain 129 mice must be extremely uncommon, since we have not observed them except in tumorous glands. Suggestive evidence, but not final proof, has been presented here that testicular teratomas originate within the tubules of fetal mouse testes, in all likelihood, from germinal cells. We are currently examining sections of fetal testes, to determine whether or not the teratomas in strain 129 mice develop within the testicular tubules.

It has been emphasized previously that the testicular teratomas in weaning-age strain 129 mice occur on the left side about 3 times more frequently than on the right (*1*, *2*), which indicates that a nongenetic environmental influence may be important in their etiology. A similar laterality preference shown by the early tumors indicates that the environmental influence may affect the initiation of carcinogenesis. According to Willis (*26*), most descriptions of human testicular teratomas show a predominance of right-sided growths, and most are probably congenital. It is possible that prenatal environmental influences may be significant in teratocarcinogenesis in the human being and in the mouse.

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PLATES

526279—59——7

PLATE 120

FIGURE 1.—Early teratoma (*encircled*) in testis of 0-day-old mouse, showing group of embryonal cells. *Note* lack of epithelial formation. For higher magnification, *see* figure 17.

FIGURE 2.—Teratoma (*encircled*) in testis of 0-day-old mouse, showing incipient epithelium formation (*arrows*). For higher magnification, *see* figure 18.

FIGURE 3.—Teratoma (*encircled*) in testis of 0-day-old mouse, showing primitive epithelium formation. For higher magnification, *see* figure 20.

FIGURE 4.—Teratoma (*encircled*) in testis of 0-day-old mouse, showing epithelial vesicle enclosing blood and cellular debris.

FIGURE 5.—Teratoma in testis of 1-day-old mouse, showing epithelial vesicle with cellular debris.

FIGURE 6.—Teratoma in testis of 2-day-old mouse, showing 2 foci. *Note* extravasated blood and cellular debris, and in focus at *right*, direct continuity between seminiferous tubule and teratomatous epithelium. For higher magnification, *see* figure 53.

FIGURE 7.—Teratoma in testis of 2-day-old mouse, showing 3 foci (*encircled*). *Note* epithelial vesicles containing blood and cellular debris.

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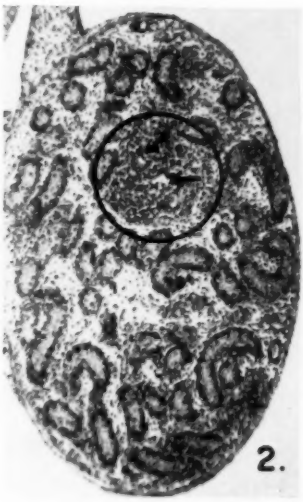
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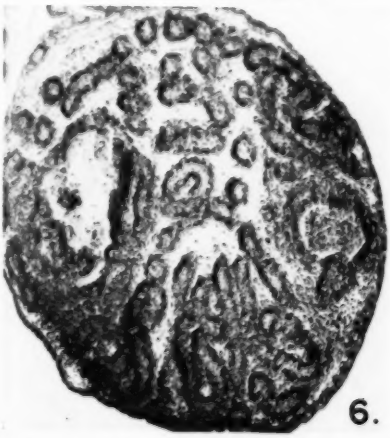
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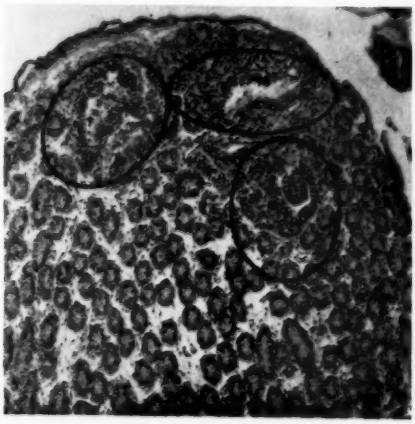


PLATE 121

FIGURE 8.—Teratoma in testis of 3-day-old mouse. *Note* ectodermal epithelium with well-defined internal and external limiting membranes enclosing blood and cellular debris.

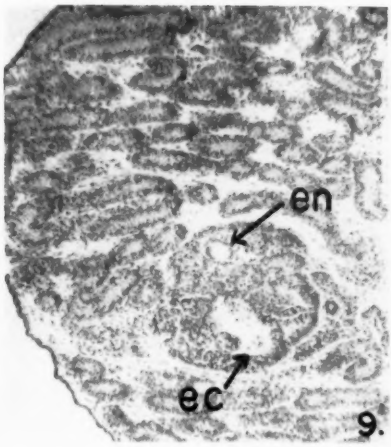
FIGURE 9.—Teratoma in testis of 3-day-old mouse. *Note* ectodermal (ec) and endodermal (en) epithelium.

FIGURE 10.—Teratoma in testis of 3-day-old mouse, showing ectodermal (ec) and endodermal (en) epithelium enclosing blood and cellular debris.

FIGURE 11.—Teratoma in testis of 4-day-old mouse, showing ectoderm (ec) and endoderm (en); presents a picture similar to mesoderm formation (mes) at primitive streak stage of normal mouse development. For higher magnification, *see* figure 26.

FIGURE 12.—Teratoma in testis of 4-day-old mouse, showing 3 primary germ layers and continuity between germinal epithelium and tumorous elements (*arrow*).

FIGURE 13.—Teratoma in testis of 4-day-old mouse, showing neuroepithelium (ne), endoderm (en), and mesoderm (mes).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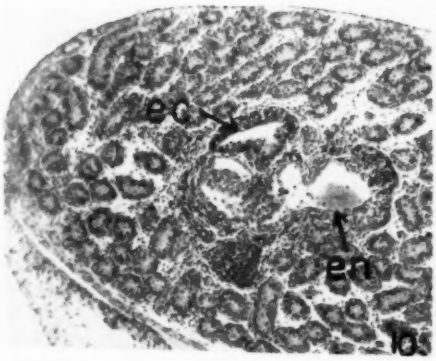
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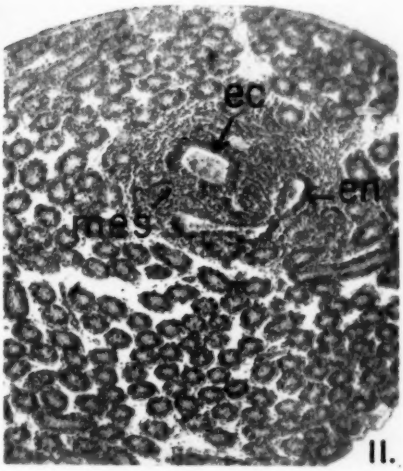
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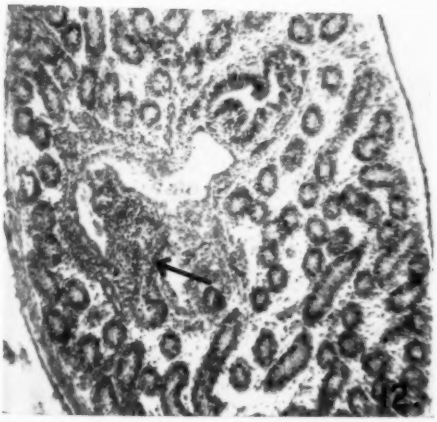
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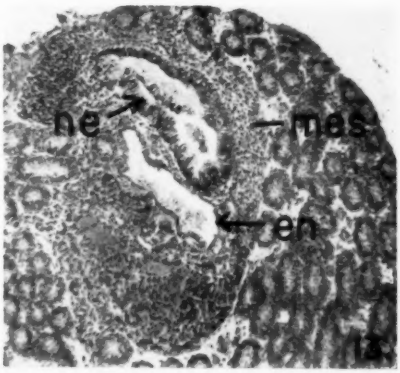
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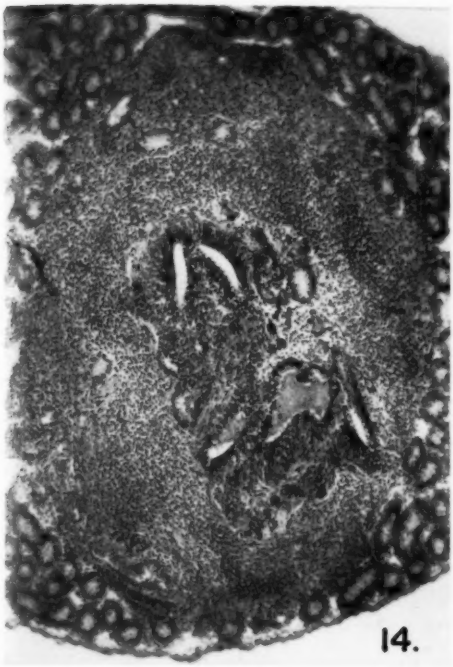
PLATE 122

FIGURE 14.—Teratoma in testis of 5-day-old mouse, showing large area of embryonic mesenchyme and early neuroepithelium.

FIGURE 15.—Teratoma in testis of 5-day-old mouse, showing heightened neuroepithelium.

FIGURE 16.—Teratoma in testis of 6-day-old mouse.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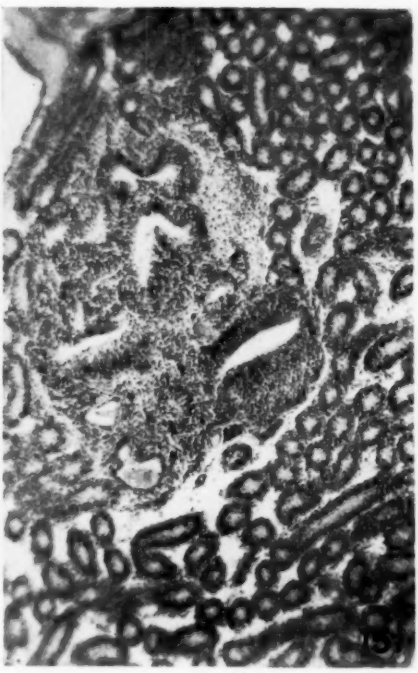
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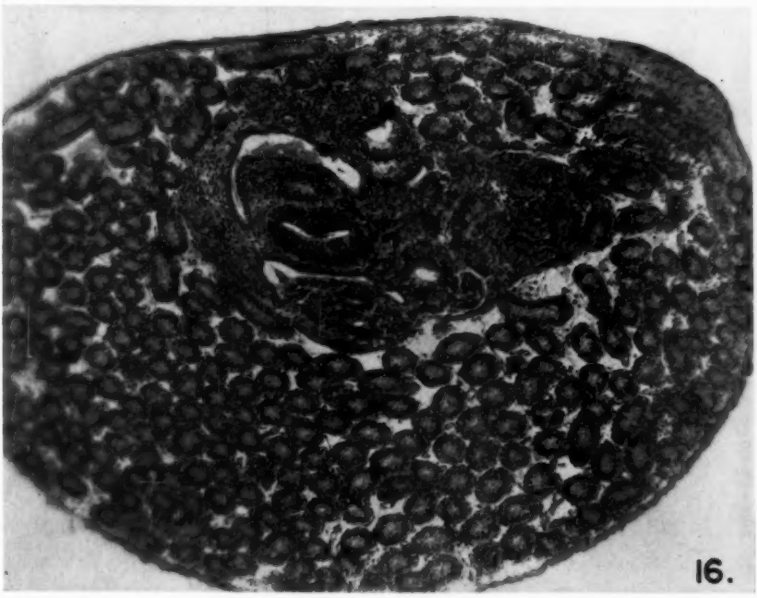
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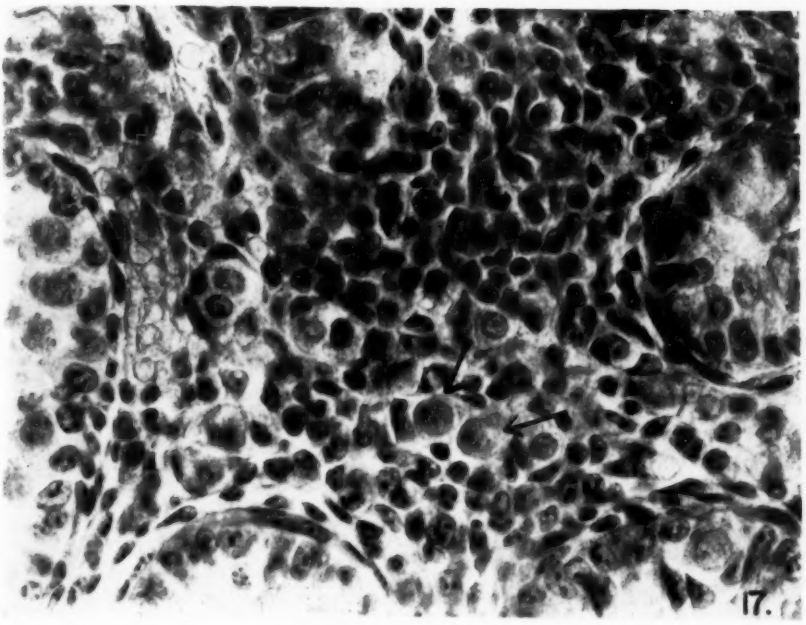
PLATE 123

FIGURE 17.—Teratoma in testis of 0-day-old mouse. *Note* ectopic germ cells (*arrows*) and lack of epithelium formation.

FIGURE 18.—Teratoma in testis of 0-day-old mouse. *Note* incipient epithelium formation (*arrows*) and ectopic germ cells (eg).



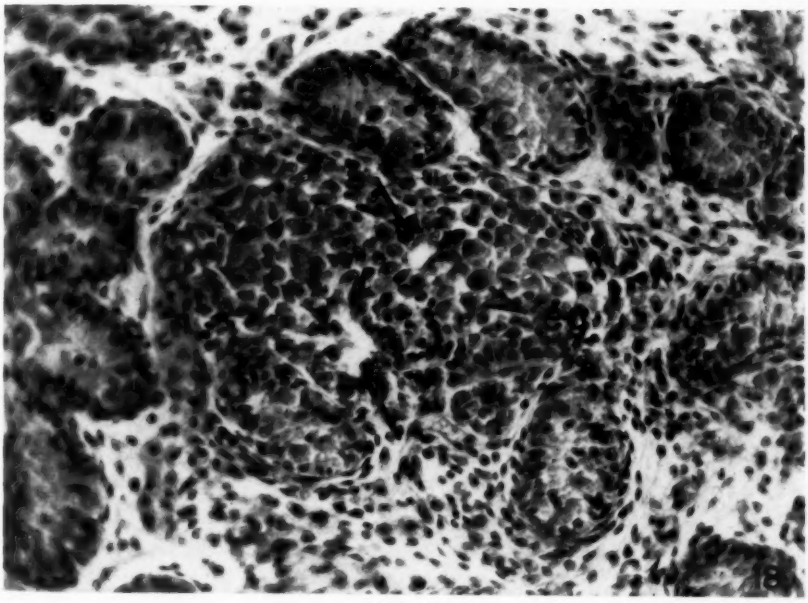
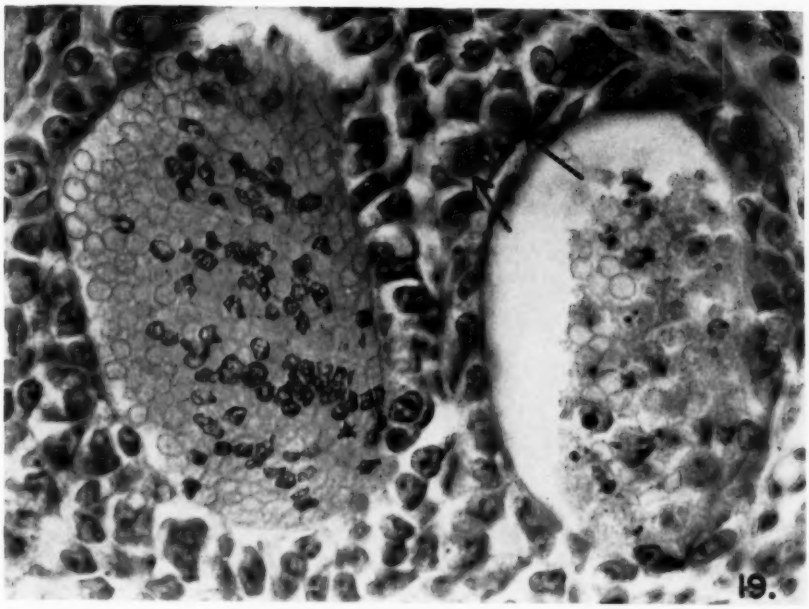


PLATE 124

FIGURE 19.—Teratoma in testis of 0-day-old mouse, showing 2 vesicles containing extravasated blood and cellular debris. *Note* ectopic germ cells (*arrows*).

FIGURE 20.—Higher magnification of figure 3, showing epithelial formation around red blood cells.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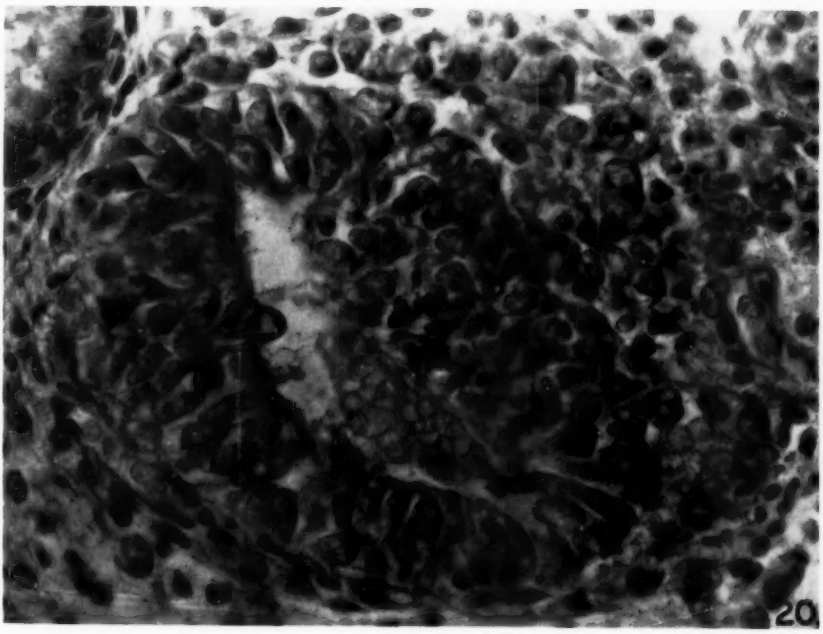
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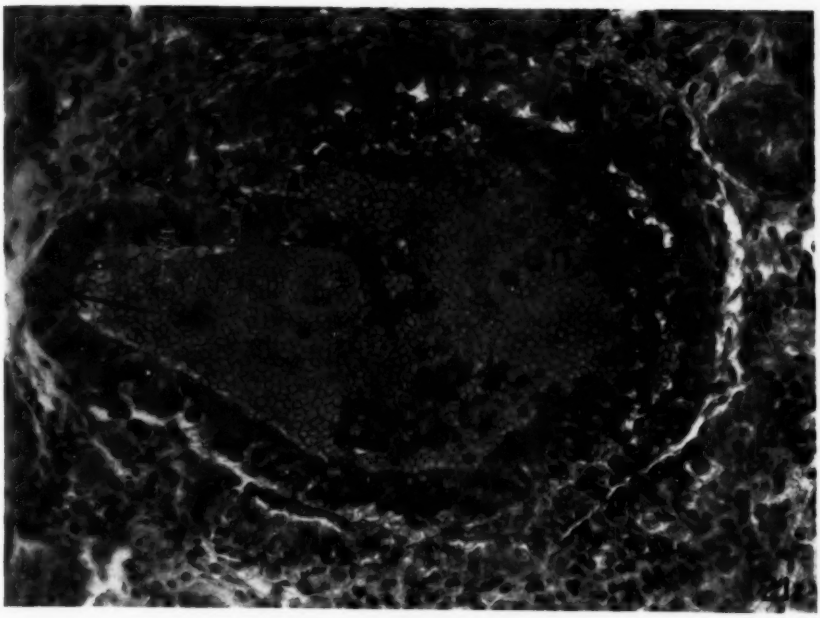
PLATE 125

FIGURE 21.—Teratoma in testis of 0-day-old mouse, showing ectoderm (ec) and endoderm (en).

FIGURE 22.—Teratoma in testis of 3-day-old mouse, presenting picture similar to mesoderm formation (*arrow*) at primitive streak stage of normal mouse development.



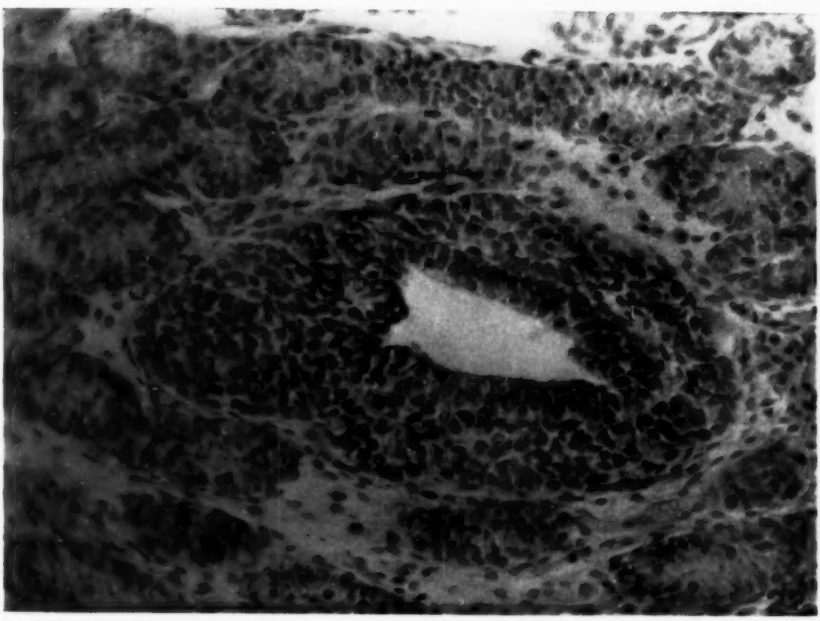
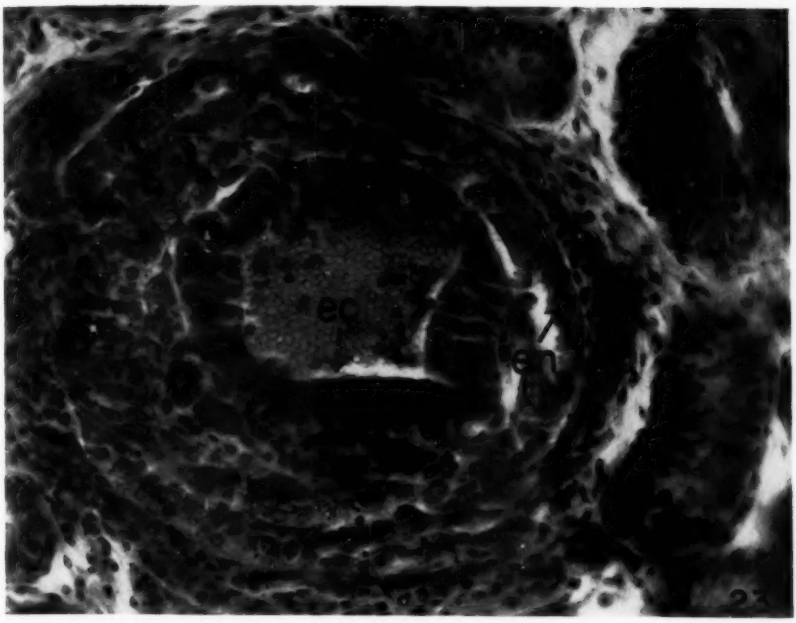


PLATE 126

FIGURE 23.—Teratoma in testis of 3-day-old mouse, showing ectodermal vesicle (ec) containing blood and cellular debris, and endodermal (en) epithelium.

FIGURE 24.—Higher magnification of figure 10 (3 days), showing ectodermal (ec) and endodermal (en) vesicles. *Note* deteriorating cells in endodermal vesicle.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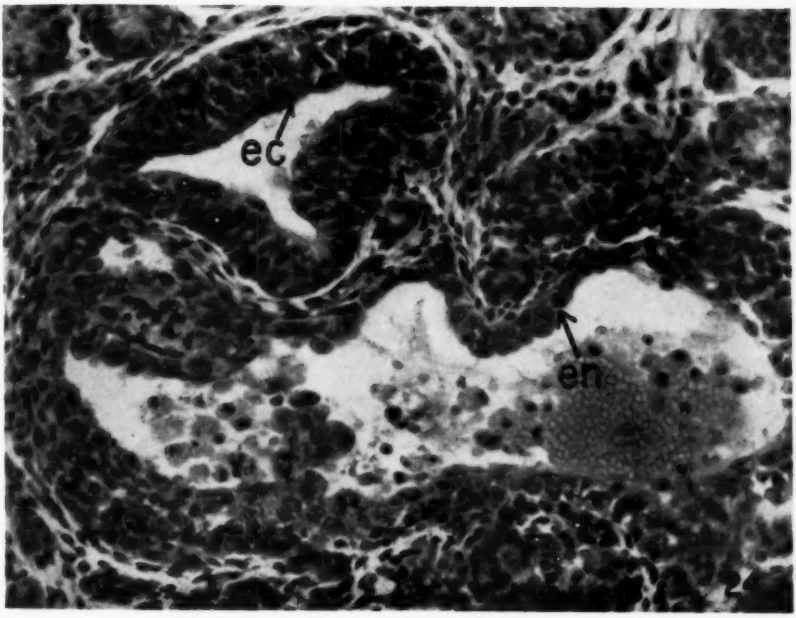
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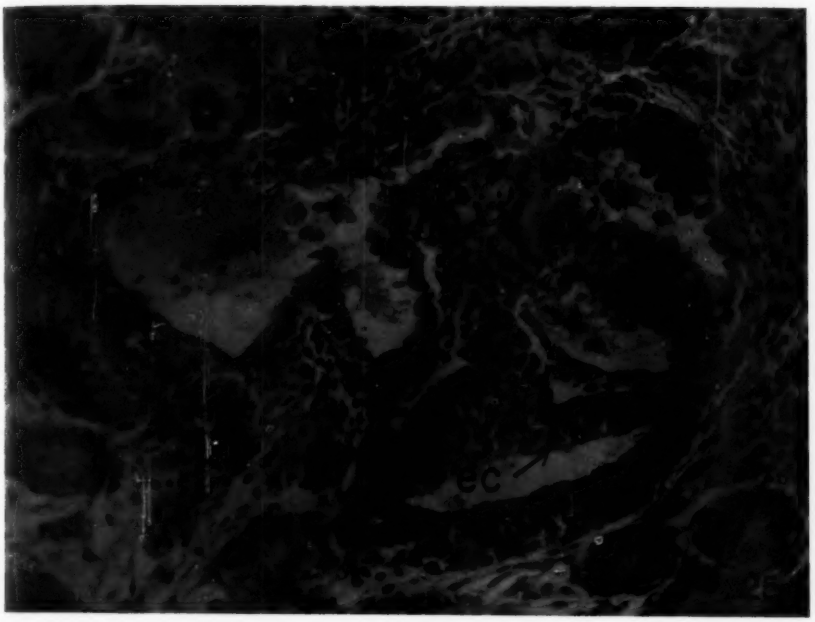
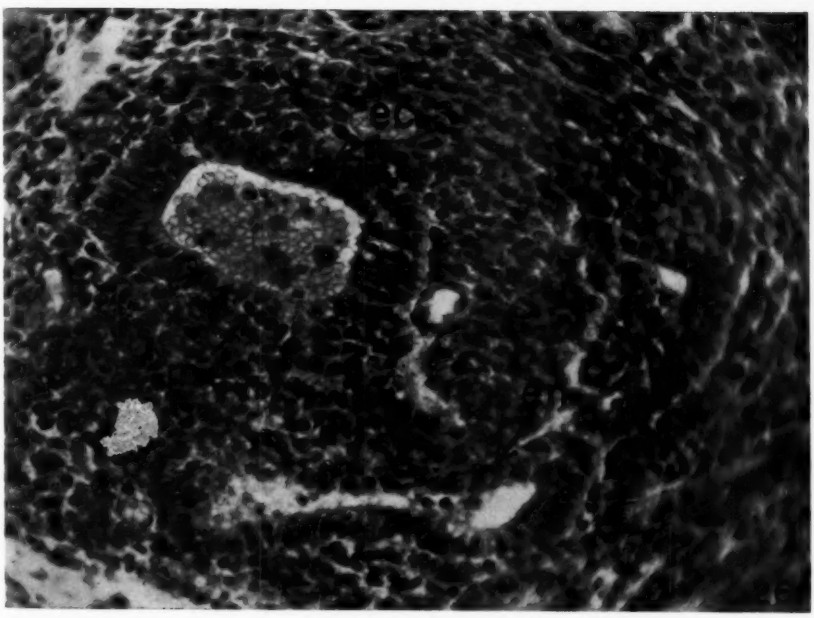
PLATE 127

FIGURE 25.—Teratoma in testis of 3-day-old mouse, showing ectodermal (ec) vesicle and endodermal (en) vesicle containing blood and cellular debris.

FIGURE 26.—Teratoma, 4 days, showing ectodermal (ec) vesicle containing blood and cellular debris and area (mes) resembling primitive streak of normal mouse embryos.





STEVENS

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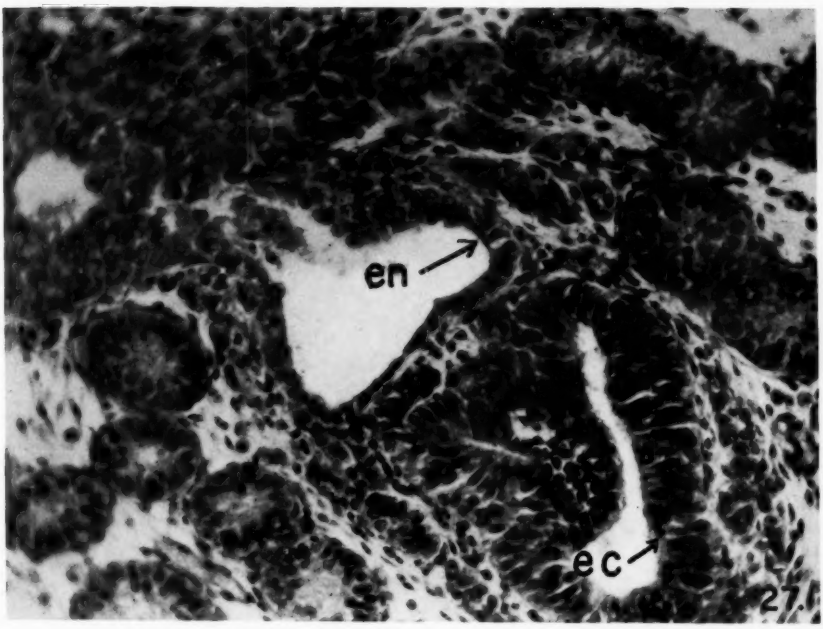
PLATE 128

FIGURE 27.—Teratoma, 4 days, showing ectoderm (ec) resembling early neuroepithelium and endoderm (en), with well-defined internal and external limiting membranes.

FIGURE 28.—Teratoma, 4 days, showing ectoderm (ec) similar to neuroepithelium and endodermal vessicle (en).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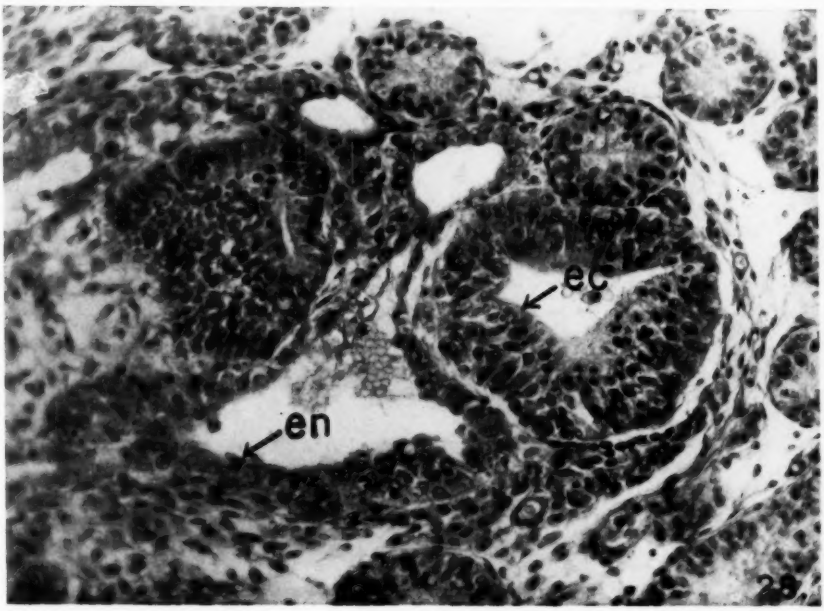
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PLATE 129

FIGURE 29.—Teratoma, 4 days, showing neuroepithelium and endodermal vesicle.

FIGURE 30.—Teratoma, 4 days, showing neuroepithelium and endodermal vesicle.

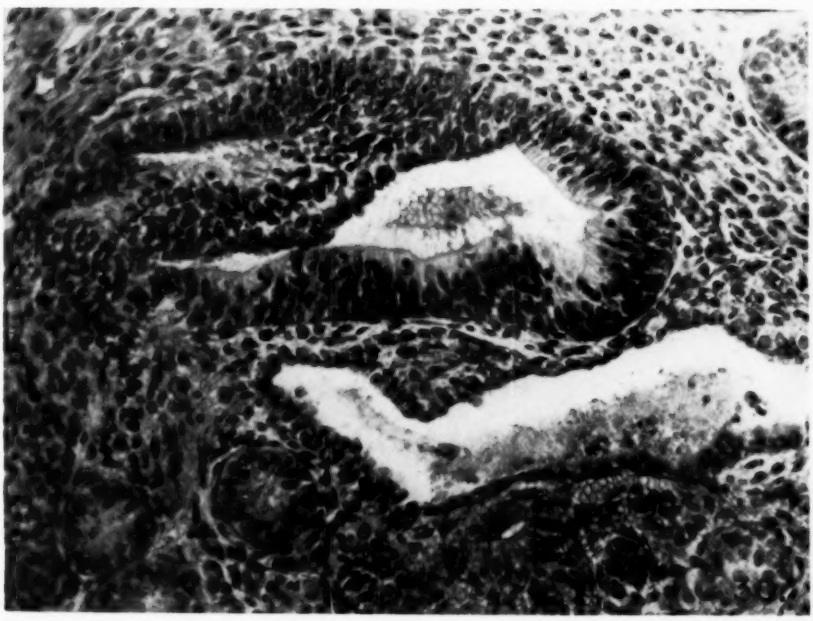


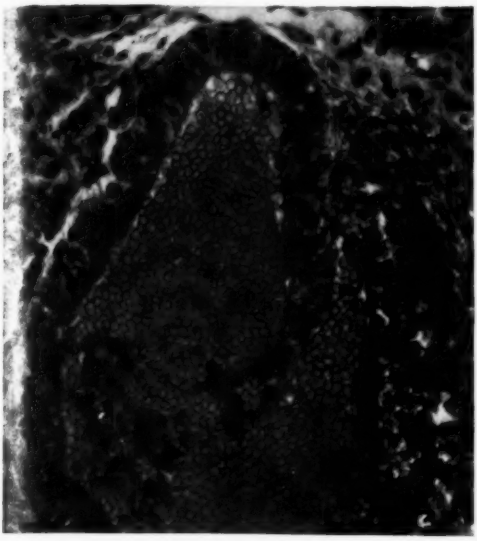
PLATE 130

FIGURE 31.—Ectodermal epithelium in teratoma of a 0-day-old mouse. *Note* single layer of columnar cells competent to form neuroepithelium.

FIGURE 32.—Neuroepithelium in teratoma of a 6-day-old mouse. *Note* resemblance to neural tube with many layers of cells and ependymal layer abutting on internal limiting membrane.

FIGURE 33.—Neuroepithelium in teratoma of a 9-day-old mouse. *Note* ependymal, mantle, marginal layers, and cluster of differentiating neuroblasts (*encircled*).

FIGURE 34.—Neural tube and bundle of nerve fibers (*arrow*) in teratoma of 6-day-old mouse.



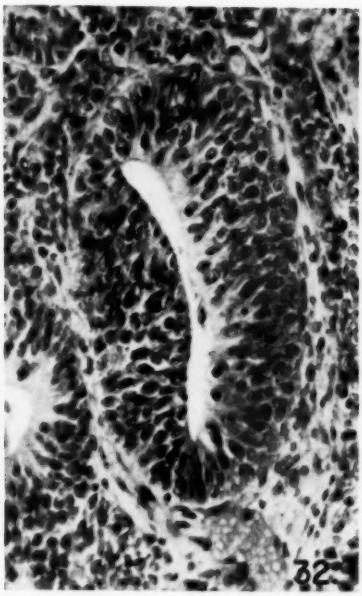

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PLATE 131

FIGURE 35.—Esophageal epithelium in 9-day-old mouse embryo.

FIGURE 36.—Hind gut and neural groove of 9-day-old mouse embryo.

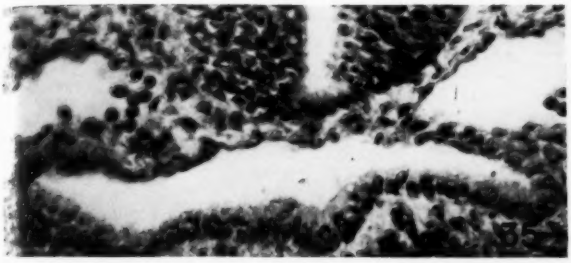
FIGURE 37.—Endodermal vesicle in teratoma of 8-day-old mouse. *Note lower*, epithelium lacks internal limiting membrane and resembles pharyngeal epithelium, and *upper*, epithelium resembling alimentary tract with distinct internal and external limiting membranes.

FIGURE 38.—Vesicle in teratoma of 8-day-old mouse, showing ciliated epithelium continuous with more primitive cells.

FIGURE 39.—Vesicle in teratoma of 9-day-old mouse. *Note* pseudostratified ciliated epithelium (*lower*) continuous with more primitive cells.

FIGURE 40.—Ciliated epithelium in teratoma of 11-day-old mouse. *Note* continuity with more primitive epithelium.

FIGURE 41.—Pseudostratified ciliated epithelium in teratoma of 11-day-old mouse. *Note* goblet cells and continuity with more primitive epithelium.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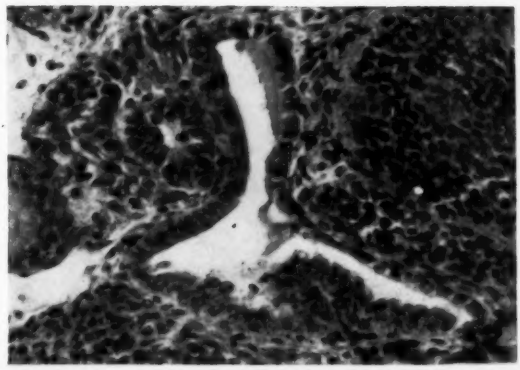

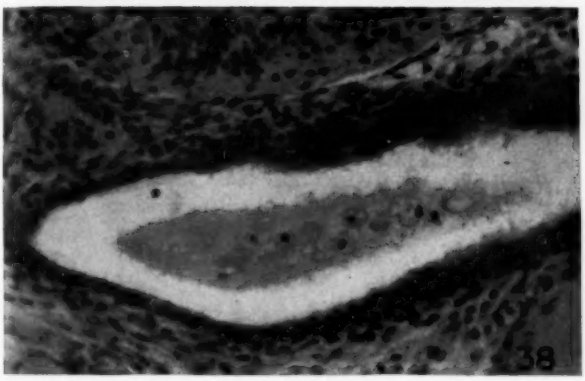
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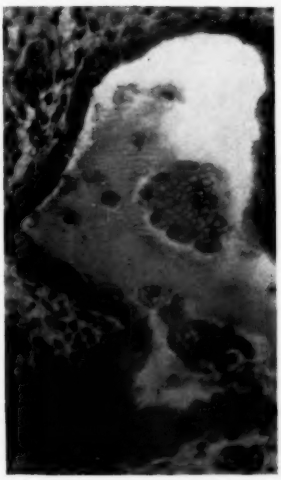

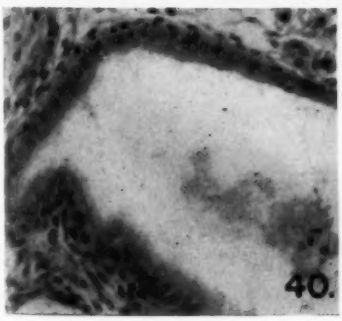
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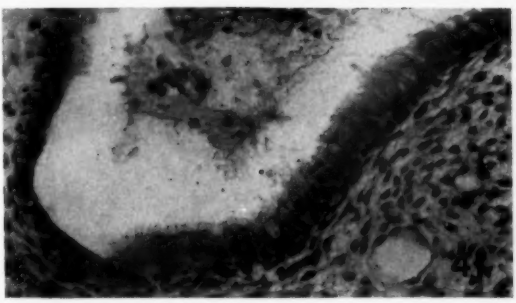
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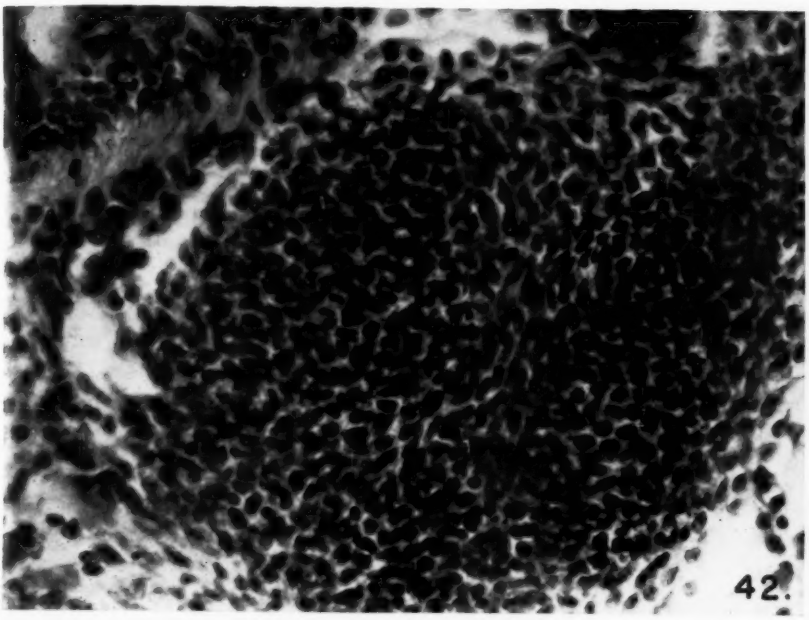
PLATE 132

FIGURE 42.—Nodule of precartilage cells in teratoma of 7-day-old mouse.

FIGURE 43.—Nodule of precartilage cells more mature than those in figure 42, in teratoma of 7-day-old mouse.

FIGURE 44.—Nodule of cartilage cells with matrix in teratoma 7-day-old mouse.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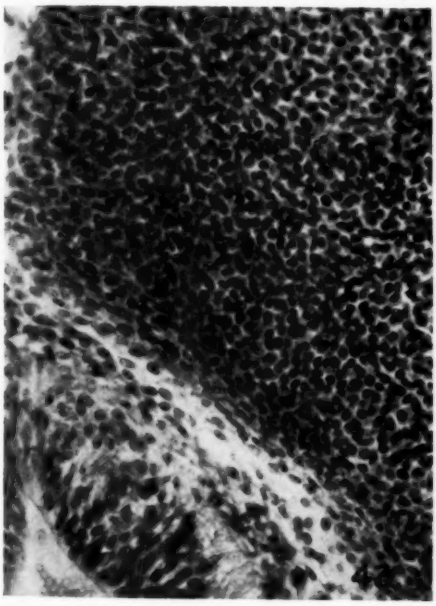


PLATE 133

FIGURE 45.—Notochordal tissue embedded in cartilage in teratoma of 9-day-old mouse.

FIGURE 46.—Cluster of vacuolated notochordal cells embedded in cartilage in teratoma of 10-day-old mouse.

FIGURE 47.—Ossification at periphery of nodule of cartilage in teratoma of 11-day-old mouse.

FIGURE 48.—Red bone marrow in teratoma of 18-day-old mouse.

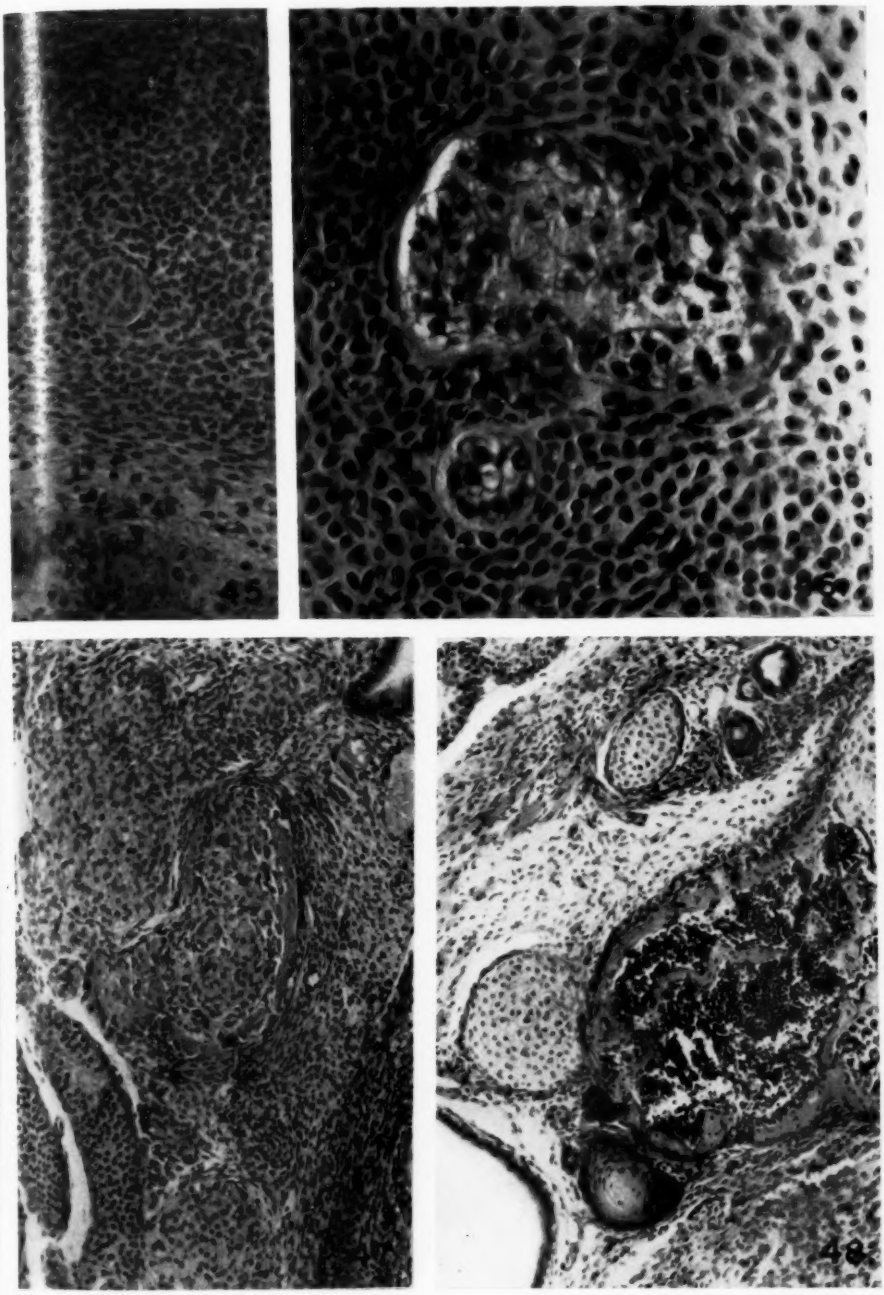


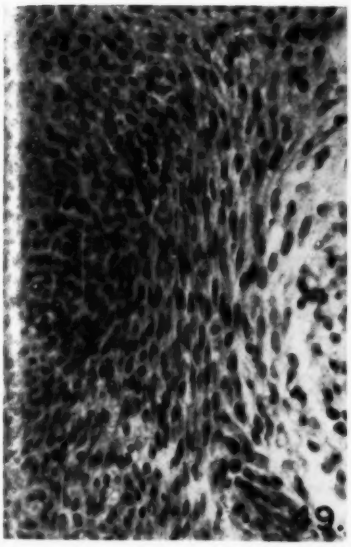
PLATE 134

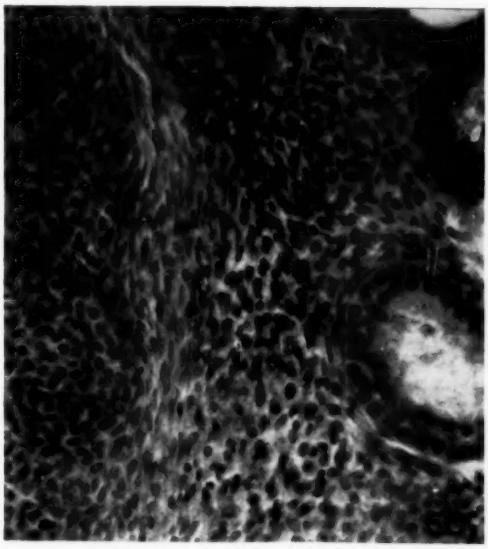
FIGURE 49.—Elongated myoblasts adjacent to precartilage cells in a teratoma of 7-day-old mouse.

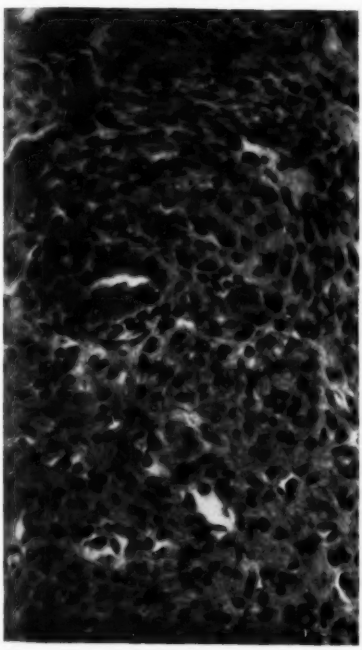
FIGURE 50.—Myoblasts developing near precartilaginous nodule in teratoma of 10-day-old mouse. At *right* are testicular tubules.

FIGURE 51.—Fetal cardiac muscle in teratoma of 6-day-old mouse.

FIGURE 52.—Striated muscle, cartilage, bone marrow, fat, and glandular structure in teratoma of 18-day-old mouse.







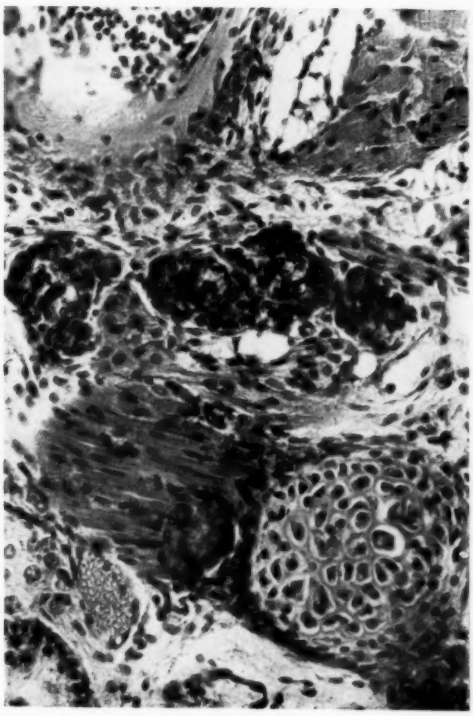


PLATE 135

FIGURE 53.—Continuity of germinal epithelium (ge) and teratoma in testis of 2-day-old mouse.

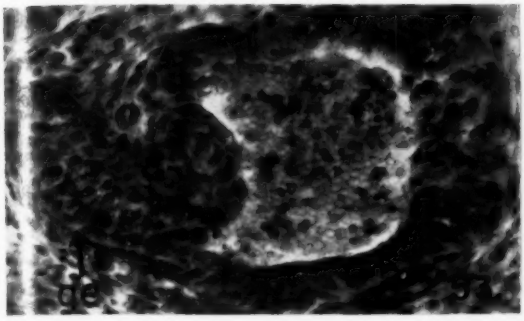
FIGURE 54.—Continuity of germinal epithelium (ge) and teratoma in 0-day-old mouse.

FIGURE 55.—Germinal epithelium (ge) continuous with teratoma in testis of 7-day-old mouse.

FIGURE 56.—Teratomatous epithelium (t) within testicular tubule of 8-day-old mouse.

FIGURE 57.—Early neuroepithelium (t) continuous with germinal epithelium (ge) in testis of 7-day-old mouse.

FIGURE 58.—Early neuroepithelium (t) in testicular tubule of 10-day-old mouse.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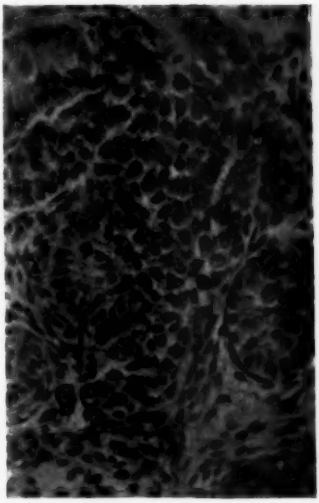

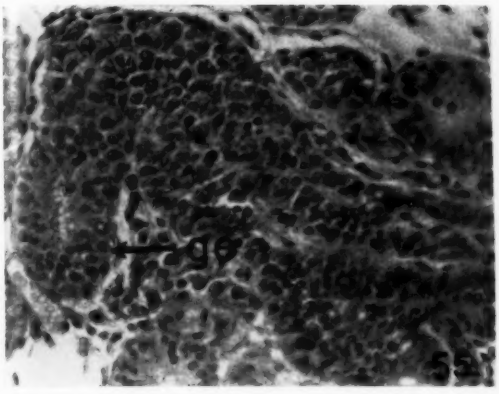
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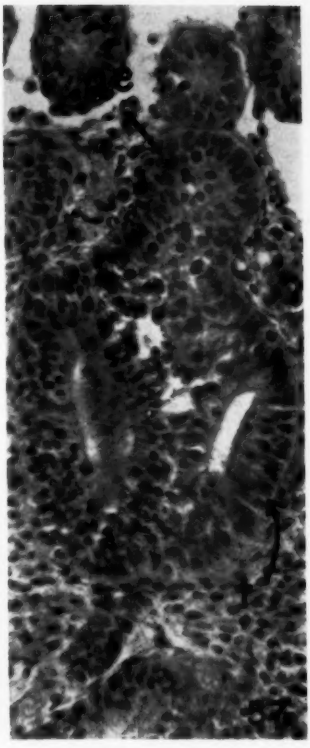

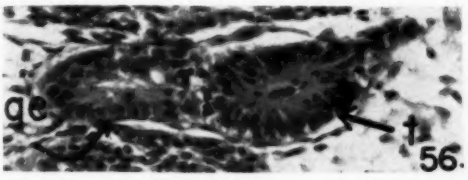
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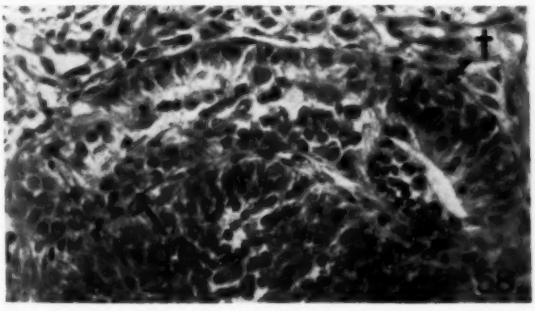
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PLATE 136

FIGURE 59.—Longitudinal section of 6-day-mouse embryo. *Note* multilayered ectodermal vesicle (ec) enclosed by simple endodermal epithelium (en).

FIGURE 60.—Embryoid body in transplantable teratoma (subline 402A VI). *Note* ectodermal vesicle enclosed by single layer of endodermal cells. Also *note* that embryoid body is surrounded by fluid.

FIGURE 61.—Embryoid body in retroperitoneal metastatic growth of testicular teratoma.

FIGURE 62.—Free-floating embryoid bodies in ascitic fluid, resulting from intraperitoneal transplantation of subline 402A VI.

FIGURE 63.—Embryoid bodies in ascitic fluid of mouse with intraperitoneal graft of transplantable teratoma, subline 402A VI.

FIGURE 64.—Embryoid body in subcutaneous graft of homogenized transplantable teratoma, subline 402A VI.

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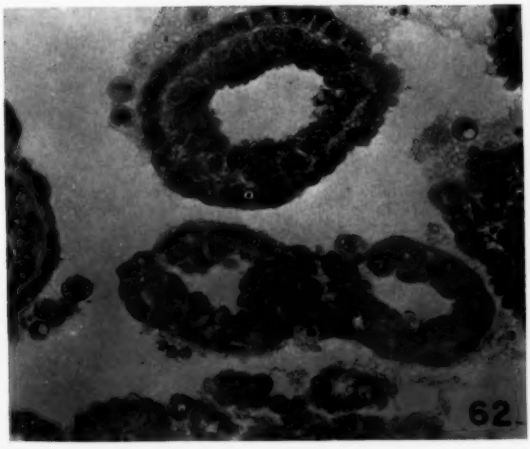
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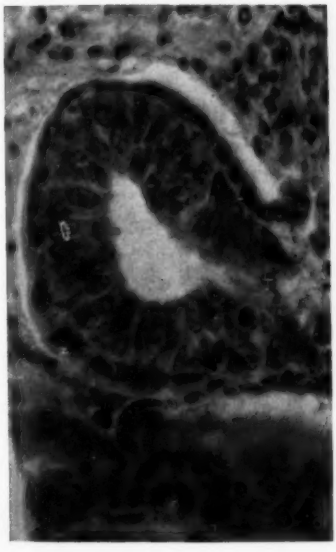

Genetic Determination of Sex-Specific Antigens [1,2]

ALLEN S. FOX,[3] *Department of Agricultural Chemistry, Michigan State University, East Lansing, Michigan*

SUMMARY

The results of an immunogenetic analysis of the effects of the X and Y chromosomes on tissue antigens (proteins) in *Drosophila melanogaster* are presented, and are used to formulate by analogy the genetic problems involved in the analysis of sex-specific antigens, particularly those connected with the Eichwald-Silmser effect in mice (rejection of male isografts by females). In *Drosophila*, 2 male-specific antigens (♂-1 and ♂-2) and 2 female-specific antigens (♀-1 and ♀-2) are demonstrated by means of agar-diffusion techniques. These differences between males and females are attributable to the difference in number of X chromosomes (1 in males and 2 in females) rather than to the presence or absence of the Y chromosome, thus suggesting involvement of the sex-determining genes carried by the X. The heterochromatic Y chromosome has no detected effect on the antigenic specificity of proteins, but it does exert a maternal influence on the immunological (precipitating) and physical properties of at least one protein (Y-1). The presence or absence of a Y chromosome in the oocytes of a female prior to meiosis determines the properties of Y-1 produced in her progeny after fertilization. Thus, questions regarding X-chromosomal dosage, presence or absence of the Y chromosome, and matrocliny must all be answered in the genetic analysis of sex-specific antigens. Description is given of experiments designed to fulfill these requirements in the case of the Eichwald-Silmser effect.—J. Nat. Cancer Inst. 23: 1297–1311, 1959.

SOMETIME AGO (*1*) we published a note in which we discussed the genetic requirements for the detection of antigenic differences attributable to the Y chromosome. More recently (*2*) we reported antigenic differences between males and females in *Drosophila melanogaster* not attributable to the Y chromosome, and pointed out the meaning of these observations for the genetic interpretation of the Eichwald-Silmser (*3, 4*) phenomenon—rejection of male isografts by females in mice. Since discussion of this phenomenon has continued (*5–9*), in most of which it is assumed that the phenomenon is attributable to Y-linked histocom-

[1] Received for publication May 25, 1959.

[2] Supported by research grants from the National Institutes of Health (C-2440) and from the All-University Research Committee, Michigan State University.

[3] The assistance of Sei-Byung Yoon and C. G. Mead is gratefully acknowledged.

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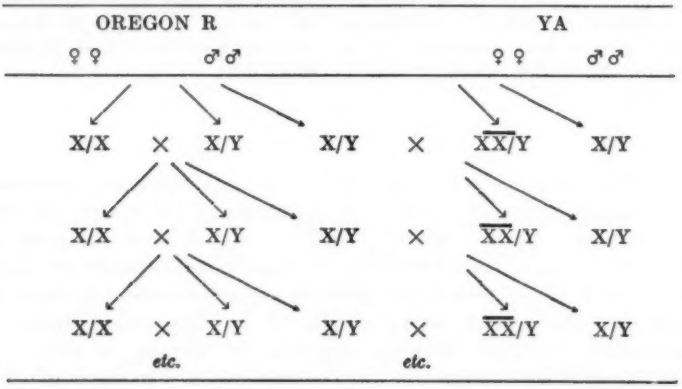
patibility genes, it seems important to report further details of our investigations of the genetic determination of sex-specific antigens in *D. melanogaster*. These results may be taken as a model of the problems involved in the analysis of the Eichwald-Silmser effect. They do not consititute an analysis of that effect *per se*, but they serve to formulate by analogy the genetic problems involved therein, and illustrate some of the complexities which could be encountered.

MATERIALS AND METHODS

This analysis of sex-specific antigens in *Drosophila melanogaster* has involved the use of two coisogenic stocks, Oregon R and YA. The Oregon R stock (series I), rendered isogenic some years ago by Dr. Jack Schultz, has since been maintained through approximately 200 generations of brother × sister matings. Females are XX and males are XY. The YA stock was derived in such a way as to possess the same autosomes and Y chromosome as the Oregon R stock. YA females, however, possess attached-X chromosomes, marked with the mutant *yellow* (*y*), which differ genetically from the Oregon R X chromosome. This difference has proved to have no consequences so far as the present analysis is concerned, but the attached-X condition renders YA females XXY in constitution, which is pertinent. YA males are XY. The 2 stocks were maintained according to the scheme outlined in table 1. In each generation, YA females were mated with Oregon R males, thus preventing genotypic divergence of the 2 stocks and guaranteeing that YA males and Oregon R males are genotypically identical.

TABLE 1.—Mating system used in maintaining Oregon R and YA stocks



Since the 2 stocks are coisogenic in all pertinent respects, antigenic differences distinguishing the 2 types of females from the 2 types of males are to be attributed to the difference in X-chromosome dosage (2 in fe-

males, 1 in males). Antigenic differences distinguishing Oregon R females from Oregon R males, YA females, and YA males would be attributable to the presence of the Y chromosome in the latter genotypes and its absence in the former.

In addition to the males and females of these 2 stocks, males and females of special genetic constitution have been examined. These will be described.

Details regarding the preparation of antigens and the immunization of rabbits have been given repeatedly (*10*). In brief, lyophilized flies of a given genotype are homogenized in buffered saline (2.0 gm. dry wt./100 ml.). Intraperitoneal injections of the whole homogenate serve to immunize the rabbits. The clear supernatant, obtained by centrifugation, is used as antigen in serological tests.

The Ouchterlony agar-diffusion technique (*11, 13*) has been used in this analysis. Preliminary work disclosed that the geometry illustrated in figure 4 is best suited for our system. The capacity of the wells is 0.15 ml. The *An* well is filled with antiserum and the other 3 wells with the antigen extract from different genotypes. All 4 wells are filled simultaneously, usually on successive days. Precipitate lines usually appear on the 4th or 5th day. Ten days are usually sufficient for the complete development of the plate at 25° C.

Serum absorptions are accomplished in the plate itself by means of the Björklund inhibition technique (*12*). For this purpose, absorbing antigen is allowed to diffuse into the agar from the *An* well prior to performance of the test. To increase the concentration range of the absorbing antigen it may be concentrated 10× by lyophilization. The *An* well may be refilled with absorbing antigen as many as 10 times prior to performance of the test. The serum may thus be absorbed by antigen varied over a concentration range of 100-fold. An absorbing antigen must be incapable of inhibiting the formation of a particular precipitate line over this whole concentration range before the conclusion that it lacks the corresponding antigen is accepted.

EFFECTS OF CHANGES IN X-CHROMOSOME DOSAGE

The analysis of numerous antiserums against the antigenic extracts of each of the 2 types of males (Oregon R and YA) and each of the 2 types of females has demonstrated qualitative antigenic differences between the sexes. Since the analysis of each such serum involves the preparation of numerous Ouchterlony plates, the results cannot be given in full detail. Several typical examples were given in our previous paper (*2*), and further examples will be given here.

Our previous report illustrated results demonstrating the presence in Oregon R and YA males of an antigen (♂-1) which is absent in females. The analysis of additional antiserums has disclosed the presence of another such male-specific antigen, ♂-2. Figure 1 contains the photograph

of a plate illustrating the basis of this conclusion, and an explanatory diagram is contained in figure 2. The serum used in this instance was anti-YA ♂♂; *i.e.*, it was prepared by the immunization of a rabbit with the antigenic extract of YA males (X/Y). The antigen wells of the plate were filled with the antigenic extracts of the genotypes indicated in figure 2; the homologous extract was used in well 1. Lines 1 through 4 represent antigenic components present in all 3 genotypes. The significance of line Y-1 will be discussed. The lines labeled ♂-1 and ♂-2 represent the 2 male-specific components, which are clearly present in YA males (X/Y) and Oregon R males (X/Y) but not in Oregon R females (X/X). Plates prepared with YA females ($\overline{XX}$/Y) disclose that they too lack ♂-1 and ♂-2.

Similar results have been obtained with other anti-YA ♀♀ serums and with anti-Oregon R ♂♂ serums. Not unexpectedly, some anti-♂♂ serums lack antibodies to these male-specific components, others possess antibodies to only one of the two, and the number of common components varies from serum to serum. However, no anti-♀♀ serum has ever contained antibodies to either ♂-1 or ♂-2.

Björklund inhibitions confirm that this difference between the sexes is qualitative in nature. Three or 4 doses (0.15 ml. each) of either YA ♂♂ or Oregon R ♂♂ antigenic extracts (concentrated 10× by lyophilization), when allowed to diffuse from the *An* well prior to the performance of the test, serve to inhibit completely the formation of all precipitate lines exhibited in figure 1. One or 2 doses of inhibiting antigen serve to shift the position of the lines progressively toward the *An* well. Similar inhibition with concentrated YA ♀♀ or Oregon R ♀♀ antigenic extract serves to remove lines 1 through 4, but the lines attributable to ♂-1 and ♂-2 remain unchanged in position or density. This result is not altered even when 10 inhibiting doses of female antigen are used. The presence of ♂-1 or ♂-2 in female extracts would result, under these circumstances, in either the inhibition of the corresponding lines or, if present in limited concentration, in a shift in their position toward the *An* well (*13*). The absence of such an effect, and the absence of antibodies to ♂-1 and ♂-2 in anti-♀♀ serums, strongly indicates the absence of these antigens in females. Results similar to those described here have been illustrated previously (*2*).

Our previous report also demonstrated the existence of 2 female-specific antigens (♀-1 and ♀-2), present in Oregon R and YA females but not in males. As with the male-specific components, the absence of the corresponding antibodies in anti-♂♂ serums and the results of Björklund inhibitions demonstrate that this also is a qualitative difference. All in all, therefore, 4 sex-specific antigens have been identified, 2 in males and 2 in females. Their distribution in the Oregon R and YA stocks is summarized in table 2.

Antigenic differences among these genotypes with respect to the 2 male-specific components (♂-1 and ♂-2) and the 2 female-specific components (♀-1 and ♀-2) are clearly not related to the presence or absence

TABLE 2.—Distribution of specific antigens in Oregon R and YA stocks

Antigen	Oregon R		YA	
	X/X	X/Y	$\overline{XX}$/Y	X/Y
♂-1	—	+	—	+
♂-2	—	+	—	+
♀-1	+	—	+	—
♀-2	+	—	+	—
Y-1	Complete	Complete	Incomplete	Incomplete

of the Y chromosome. They are related instead to the number of X chromosomes. Flies with one X possess the 2 male-specific components, while flies with 2 X's possess the 2 female-specific components. In other words, a change in X-chromosome dosage from 1 to 2 (in the presence of 2 sets of autosomes) results in a qualitative shift in antigenic pattern. The physiological mechanisms responsible for this shift would better be discussed elsewhere, but the situation is clearly similar to that involved in sex determination ($1X:2A=$♂, $2X:2A=$♀). Indeed, the sex-determining loci themselves may be the genes responsible for the production of these antigens, which might then be considered products of sex differentiation.

The conclusion that the Y chromosome has nothing to do with these differences is confirmed by the further results summarized in table 3. These results were made possible by the use of a variety of normal and modified sex chromosomes, including: (1) the normal X chromosome (X); (2) the normal Y chromosome (Y); (3) attached-X chromosomes ($\overline{XX}$); (4) attached X-Y chromosomes (X·Y); (5) the short arm of the Y chromosome attached to an intact X chromosome (X·Y^S); (6) the long arm of the Y attached to an intact X (X·Y^L). The long and short arms of the Y have been identified both cytologically and by their content of male-fertility genes. The symbol O indicates the absence of a second sex chromosome. The full genotypes, in *Drosophila* symbolism, are given in the second column of the table.

Table 3.—Effects of X-chromosome dosage on sex-specific antigens

Sex chromosomes	Genotype	Antigens			
		♂-1	♂-2	♀-1	♀-2
X/X	+/+	—	—	+	+
$\overline{XX}$/Y	y-/Y	—	—	+	+
X·Y^S/X·Y^S	y·Y^S/y·Y^S	—	—	+	+
X·Y^L/X·Y^L	y·Y^L/y·Y^L	—	—	+	+
X/X·Y	+/Y^S·XInEnBy·Y^L	—	—	+	+
X/Y	+/Y	+	+	—	—
X/O	+/O	+	+	—	—
X·Y^S/Y	y·Y^S/Y	+	+	—	—
X·Y^L/Y	y·Y^L/Y	+	+	—	—
X·Y/O	Y^S·XInEnBy·Y^L/O	+	+	—	—

The striking feature of these results is that individuals possessing 2 X chromosomes always lack ♂-1 and ♂-2 and possess ♀-1 and ♀-2 regardless of their Y-chromosomal constitution, while the reverse is true of individuals possessing only 1 X chromosome.

It should be noted that 3 different X chromosomes have been used in these experiments. The normal X chromosome of the isogenic Oregon R stock is present in X/X, X/Y, X/O, and X/X·Y flies. The X in $\overline{XX}$/Y, X·Y^s/Y·Y^s, X·Y^L/X·Y^L, X·Y^s/Y, and X·Y^L/Y flies is the X chromosome of YA females, the genetic constitution of which has been described. The X in X·Y (X/X·Y and X·Y/O flies) carries a large inversion (In En), the Bar duplication, and the mutant yellow (*y*). In spite of these genetic differences the dosage effects are the same, indicating the generality of the phenomenon.

With these results in mind, we may return to the Eichwald-Silmser phenomenon. Obviously, if we had examined only X/X and X/Y flies we could not have known whether the antigenic differences between them arose from the difference in number of X's or from the difference with respect to the Y chromosome. But this is exactly the status of the genetic analysis of the Eichwald-Silmser phenomenon. It does not matter that, unlike *Drosophila*, there is no evidence of female-specific tissue antigens in mice. Genetic analysis is concerned with differences, and in this respect the situation in mice is parallel to that in *Drosophila*, for in both cases males possess one or more antigens which females do not.

EFFECTS OF THE Y CHROMOSOME

The foregoing analysis demonstrates that the Y chromosome is in no way associated with the production of the sex-specific antigens. It should not be concluded, however, that this chromosome is without antigenic effect. It is indeed associated with component Y-1, but in a manner which raises the possibility of additional complications in the genetic analysis of the Eichwald-Silmser phenomenon.

In figure 1, component Y-1 is represented by a line of precipitate opposite well 2 (Oregon R ♂♂, X/Y) and well 3 (Oregon R♀♀, X/X), but not well 1 (YA ♂♂, X/Y). It will be recalled, however, that the serum used in this test was anti-YA ♂♂, and it may further be stated that the antibody responsible for this line was not present in the normal serum from the same rabbit. Apparently, therefore, the Y-1 antigen is present in YA ♂♂ and has induced the formation of the corresponding antibody, but fails to form a precipitate with that antibody in Ouchterlony tests. Indeed, no precipitate is formed even when the concentration of the YA ♂♂ antigenic extract is increased as much as tenfold. Furthermore, identical results are obtained with the antigenic extract of YA ♀♀, and the antibody to Y-1 is regularly observed in antiserums to Oregon R males or females as well as to YA males or females. The results are the same in all cases.

The conclusion that Y-1 is present in YA males and females is confirmed

by the results of Björklund inhibitions. Regardless of the antiserum which is used, concentrated extracts of Oregon R males or females or of YA males or females inhibit the formation of the Y-1 line. As judged by inhibiting capacity, the specificity of Y-1 is indistinguishable in the 2 stocks. Extensive analysis, the details of which will be published elsewhere, strongly suggests that the difference between the 2 stocks with respect to the properties of Y-1 is not simply quantitative. In Oregon R males and females the Y-1 specificity is associated with an antigen which is capable of inducing antibody formation in the rabbit, and which is capable of uniting with and precipitating the specific antibody. In other words, Y-1 is present in the form of a "complete" antigen in flies of the Oregon R stock. In YA males and females the Y-1 specificity is associated with an antigen which is capable of inducing antibody formation, which can unite with and inhibit the specific antibody, but which does not form a precipitate with the antibody. To describe this situation we say that Y-1 is an "incomplete" antigen in the YA stock.

The difference between complete and incomplete Y-1 probably reflects a physical difference between the respective molecules of a sort not involving a difference in the configuration of their antigenic groupings but rather in their number. This could be the result of either a difference in the size of the respective molecules or in the way in which the polypeptide chain is folded. Both forms of Y-1 are nondialyzable, heat labile, and precipitated by protein precipitants (alcohol, TCA). Complete Y-1 is precipitated from crude extracts by simple dialysis against water. A physical difference between complete and incomplete Y-1 is suggested by a greater resistance of the former to inactivation during lyophilization.

From a genetic point of view, it is immediately apparent that the presence or absence of a Y chromosome in an individual has nothing to do with whether the Y-1 antigen will be complete or incomplete. Thus Oregon R and YA males are genetically identical, but the former possess complete Y-1 while the latter possess incomplete Y-1. A clue to the explanation of this paradox comes from the recognition that these 2 types of males have different mothers. The kind of Y-1 found in an individual (male or female) depends on whether its mother was an Oregon R female or a YA female. The inheritance of this difference in Y-1 is matroclinous.

The question therefore arises, in what respects do Oregon R and YA females differ? Two possibilities exist: (1) The difference between them might be one involving cytoplasmic inheritance, *i.e.*, a self-reproducing cytoplasmic system, independent of the Y chromosome, transmitted through the eggs (*14*); (2) YA females possess a Y chromosome while Oregon R females do not. Perhaps the presence or absence of a Y chromosome in the oocyte prior to meiosis determines the kind of Y-1 produced in the individual after fertilization.

In order to distinguish between these two possibilities, the matings portrayed in table 4 were performed. YA females were mated with X·Y/O males (generation 1). Their $\overline{XX}$/O daughters were also mated with X·Y/O males (generation 2), to produce $\overline{XX}$/O females and X·Y/O

males in generation 3. If the matroclinous tendency exhibited by Y-1 is attributable to cytoplasmic inheritance, flies of generation 3 should possess incomplete Y-1 since their cytoplasm is derived from YA females. If the presence or absence of a Y chromosome in the oocytes of a female determines the kind of Y-1 her progeny will possess, flies of generation 3 should possess complete Y-1. Figure 3 demonstrates that $\overline{XX}$/O females of generation 3 possess complete Y-1. The same is true of their X·Y/O brothers, and it is concluded that the presence or absence of a Y chromosome in a female determines whether her progeny will possess incomplete or complete Y-1.

TABLE 4.—Mating system used to distinguish between cytoplasmic inheritance and maternal influence of the Y chromosome as responsible for difference between complete and incomplete Y-1

Generation	Female		Male
1	$\overline{XX}$/Y(YA)	×	X·Y/0
2	$\overline{XX}$/0	×	X·Y/0
3	$\overline{XX}$/0		X·Y/0

Thus, the heterochromatic Y chromosome produces no detected antigenic effects in individuals possessing it. When present in the oocyte of a female, however, the Y chromosome induces a chain of events which is manifested in the form of the alteration of the physical properties, but not the antigenic specificity, of one or more proteins in her progeny. Some of the theoretical implications of this effect have been outlined elsewhere (*15*), but it is clearly unlike the postulated involvement of the Y chromosome in the Eichwald-Silmser phenomenon, and it could not be responsible for that phenomenon. Matrocliny in a more general sense, however, could be responsible for the differences exhibited by different inbred strains with respect to the intensity of the Eichwald-Silmser phenomenon (*16*).

DISCUSSION

Our purpose in the continuing discussion of the Eichwald-Silmser phenomenon has been a simple one (*1*). We have been concerned with gaining recognition of the fact that "differences between males and females [otherwise coisogenic] may be attributed either to the difference with respect to the presence of the Y chromosome or to the difference with respect to the number of X chromosomes." The point that we have sought to make is that unless one accepts certain subsidiary hypotheses, this statement is as applicable to the antigenic difference which is responsible for the rejection of male tissues by females as it is to any other difference between the sexes.

This is such an elementary genetic consideration as to make one wonder why it was not raised at the very beginning of the discussion of the Eichwald-Silmser phenomenon. The difficulty, I believe, stems from the fact that a particular subsidiary hypothesis *is* given almost unquestioned acceptance in the field of transplantation genetics. The hypothesis to which I refer is that which supposes that the presence of each tissue specificity (antigen) is dominant to its absence, and is the product of a single gene which produces its effect without interaction with either allelic or nonallelic genes. If this proposition is correct, the presence of one or more antigens in males but not in females would have to be attributed to one or more Y-linked histocompatibility genes, for the difference in X-chromosome dosage would have no antigenic consequences. It should be recognized, however, that the available evidence concerning the Eichwald-Silmser phenomenon is not sufficient *per se* to establish Y-linked histocompatibility genes, and that acceptance of such a conclusion is based on *a priori* acceptance of the foregoing hypothesis.

I have elsewhere (*10*) discussed evidence that the one gene-one specificity hypothesis is not universally applicable, and that it has indeed not been critically demonstrated in any case. I do not suppose that this evidence will be convincing to all workers in the field, but it is sufficiently extensive to demand that the interpretation given to each case be based on the available internal circumstances rather than on some external supposition. In the case of the Eichwald-Silmser phenomenon, this demands independent variation of the number of X chromosomes and the presence or absence of the Y. But it is just this kind of evidence that is lacking, for all individuals possessing 2 X chromosomes (females) which have been examined have lacked a Y, and all individuals with one X chromosome (males) have possessed a Y. The 2 sources of variability have not been separated.

In view of these considerations, it is clear that evidence demonstrating that the rejection of male tissues by females in mice is attributable to Y-linked histocompatibility genes is not yet forthcoming. Such work as that of Feldman (*5*), Casey *et al.* (*8*) or of Krohn (*9*) pertains either to the immunological basis of the rejection or to the universality of the phenomenon, but adds nothing to the genetic analysis. In particular, the work of Feldman (in spite of the title of his paper) constitutes an elegant demonstration of the antigenic basis of the phenomenon but cannot in any sense be taken as supporting the hypothesis of Y-linkage. The same comment applies to the work of Billingham and Silvers (*17*).

Coincidentally, the term "sex-linked" has been applied to the histocompatibility antigens responsible for this phenomenon (*18*, *19*). This term has a specific meaning in genetics; it is applied to genes located on the X chromosome (*20*). Until the genetic determination of the Eichwald-Silmser phenomenon is clarified, it would be best not to refer to it as a "sex-linked" phenomenon. Instead, I would suggest that the antigens involved be referred to as "sex-specific," thus avoiding commitment as to their genetic determination.

In point of fact, there already is available some evidence which suggests, though not conclusively, that these antigens are not the products of Y-linked loci. Short and Sobey (*16*) have suggested that differences in the frequency of rejection of male tissue by females in different inbred strains are attributable to differences in the postulated Y-linked genes. To test this possibility, they suggest that reciprocal matings be performed between inbred strains differing in this manner. The F_1 males produced in this manner would then differ with respect to the presumably different Y chromosomes, the sons of each of the reciprocal crosses having received their Y chromosomes from the paternal parent. Tests involving such reciprocal F_1 males, serving either as donors or recipients, would then serve to demonstrate whether their transplantation specificity differs, resembling in each case that of the paternal parent, and therefore attributable to differences in the Y chromosomes of the two inbred strains.

Such tests have now been performed, and in no case have the reciprocal F_1 males exhibited differences in transplantation specificity. This apparently is true both for normal tissue (*19*, *21*, *22*) and for a tumor exhibiting male isograft specificity (*23*).

At first sight such evidence would seem to contradict the Y-linked hypothesis in a conclusive manner, but we have already warned (*2*) that "... the results of such an experiment ... might be equivocable, since the Y chromosome would be present only in individuals with one X, and since different genetic backgrounds ("modifiers") might influence the frequency of rejection." Indeed, Michie and McLaren (*6*) have pointed out that the experiments of Eichwald *et al.* (*19*) indicate that the genotype of the female recipient is critical in such cases. The experiments of Zaalberg (*22*) make the same point.

A clear-cut solution of the problem of whether the Eichwald-Silmser phenomenon is attributable to Y-linked histocompatibility genes could come if XXY individuals or XO individuals were available. Such individuals might occur sporadically as a result of nondisjunction of the sex chromosomes, or they could be produced at will if either attached-X chromosomes, attached X-Y chromosomes, or a suitable Y-autosome translocation were available in the mouse. The job of deriving such chromosomes would be a formidable one, but it might be undertaken as a secondary objective of a larger program, particularly one in which radiation is being used as a mutagenic agent.

There is a more easily accomplished alternative, however, suggested by the experiments of Eichwald *et al.* (*19*), with tumors induced in males. Of 6 such tumors tested by Eichwald and his collaboratores, only one was found to carry the male factor. The other 5 were accepted by females of the inbred strain in which they originated. As suggested by Eichwald, the loss of the male factor might be the consequence of the changes in chromosome number which occur in tumors. If so, is the loss of the male specificity invariably accompanied by the loss of the Y chromosome, or is it the consequence of changes in the number of X chromosomes or

autosomes? Cytological examination of such tumors should settle the issue, and an experiment of this sort is projected for the near future.

ADDENDUM

The reports of Russell, Russell, and Gower (*24*) and of Welshons and Russell (*25*) have appeared just as the manuscript of this paper was being prepared for transmission to the editor. Combined genetic and cytological analysis has demonstrated: (1) Nondisjunction gives rise to X/O individuals in the mouse with a frequency of approximately 1 percent. (2) In contrast to the situation in *Drosophila*, such individuals are females. (3) Thus, while the Y chromosome in *Drosophila* carries no sex-determining genes, the Y chromosome of the mouse must be the bearer of male-determining factors. These findings have a direct bearing on the discussion of this paper.

In the first place, the demonstration of X/O individuals in the mouse makes possible the direct test of the Eichwald-Silmser phenomenon which has been outlined. The performance of the test would require the transfer of a sex-linked mutant like *Ta* (*Tabby*) to an isogenic background such as that of C57BL. Matings of $Ta/+ \times +/Y$ would then make possible the detection of X/O individuals (*Ta*/O). If the Y-chromosome hypothesis of the Eichwald-Silmser phenomenon is correct, the isograft specificity of such X/O's should be identical with that of normal X/X females. If the X-chromosome dosage hypothesis is correct, their isograft specificity should be the same as that of X/Y males. It is hoped that this experiment can be performed in the near future.

Beyond this, the conclusion that the Y chromosome of the mouse carries male-determining factors raises some interesting possibilities. If, as I have suggested, the sex-specific antigens are products of the sex-determining loci themselves, the Eichwald-Silmser effect could well be attributable to the Y chromosome and still be fundamentally similar in genetic determination to the sex-specific antigens of *Drosophila*.

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PLATES

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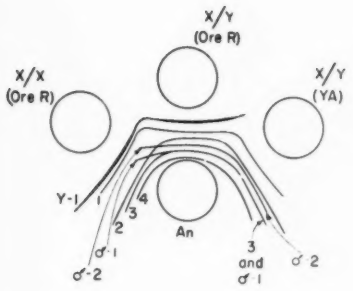
FIGURES 1 and 2.—Photograph and diagram of Ouchterlony plate developed with anti-YA♂♂ serum (*An* well). Antigen wells filled with extracts of genotypes indicated in figure 2. Lines 1 through 4: common antigenic components. Lines ♂-1 and ♂-2: male-specific components. Line Y-1: antigen exhibiting maternal influence of Y chromosome.

FIGURE 3.—Demonstration that Y chromosome exerts maternal influence on antigen Y-1. Serum: anti-YA♂♂, same as in figure 1. Antigenic extracts: Well 1, YA♀♀ ($\overline{XX}$/Y); Well 2, Oregon R ♀♀ (X/X); Well 3, $\overline{XX}$/O ♀♀ of generation 3 (*see* table 4). Y-1 is represented by the *heavy line* closest to the antigen wells. *Note* that it is incomplete opposite well 1, but complete opposite wells 2 and 3.

FIGURE 4.—Geometry of Ouchterlony agar-diffusion plates: 90-mm. petri dish, filled with 30 ml. of 2 percent agar in 0.85 percent NaCl buffered at *p*H 7.4 with 0.005 M phosphate. Dimensions in inches.

1



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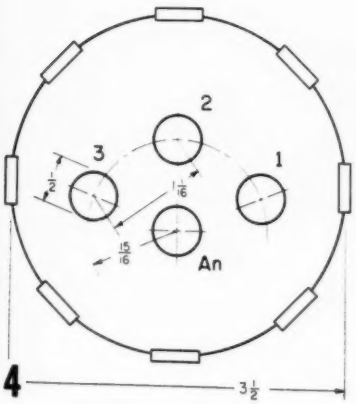

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An Examination of Some Experimental Cancer Data in the Light of the One-Hit Theory of Infectivity Titrations [1]

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SUMMARY

An investigation was made of the extent to which some previously published quantal response data, from experiments with tumor viruses, can be explained by the theory that tumor initiation is a random event. Data from experiments with the Rous sarcoma virus suggest that the chickens fell into two groups, differing widely in susceptibility. Experiments with Shope papilloma virus protein are more difficult to interpret. There were differences in susceptibility between animals, and some variation also was apparent between sites on a given animal.—J. Nat. Cancer Inst. 23: 1313–1330, 1959.

1. INTRODUCTION

THE "one-hit" or "independent action" theory of infectivity titrations with a quantal response has been described in a number of publications, *e.g.*, Peto (*1*), Moran (*2*), Armitage and Spicer (*3*), and Meynell (*4*). When various doses of infective, *i.e.*, self-reproducing particles, usually bacteria or viruses, are inoculated into groups of host organisms, such as animals, egg membranes, or plant leaves, the proportion of hosts to be infected usually increases with increasing dose. The one-hit theory, in its simplest form, postulates that for a detectable infection it is sufficient to have one particle, which for some reason or other is "effective"; that is, able to initiate an infection that can be detected. Particles act independently, and any particle chosen at random from the inoculated population has a certain probability, p, of being effective on any particular occasion. Whether a particular host is detectably infected, then, depends on whether the inoculum which it receives contains at least one effective particle. This is a matter of chance, and one can predict how the proportion of infected hosts will increase with the dose. There is thus no need to assume any variability in the susceptibility of different hosts;

[1] Received for publication May 26, 1959.

[2] Part of the work was done during my appointment in 1957–58 as Visiting Scientist at the National Cancer Institute, Bethesda, Md.

[3] I am grateful to Dr. W. Ray Bryan for putting unpublished data at my disposal and for helpful comments on this work.

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even if all hosts were absolutely uniform, the random distribution of effective particles would ensure that some hosts receiving a particular dose would be infected, while others would remain uninfected.

However, as a modification of this simple theory we may assume some variability in the susceptibility of different hosts. This is taken to mean that p varies from host to host—the most susceptible hosts having the highest values of p for the virus population under consideration, the most resistant hosts having the lowest values.

In relation to the production of virus tumors, p would be taken to be the probability that a randomly chosen virus particle initiated an infection resulting in a detectable tumor. Apart from a brief discussion at the end of the paper we are not concerned with the mechanism of tumor initiation by the virus or with the time relationships involved. No attempt is made to examine thoroughly other theories explaining the nature of the dose-response curve; the main aim here is to see what sort of modifications of the simple one-hit theory would be required to explain certain experimental results.

Suppose that, at a particular dose level, particles are inoculated into a group of host organisms. If p is the same for each host, the proportions of uninfected and infected hosts are, respectively,

$$P = e^{-\lambda p} \tag{1}$$

and

$$Q = 1 - e^{-\lambda p}. \tag{2}$$

If p varies from host to host, with cumulative distribution function $F(p)$, the proportion of uninfected hosts takes the form

$$P = \int_0^1 e^{-\lambda p} dF(p). \tag{3}$$

The right side of equation (3) may be written as $\int_0^1 e^{-\lambda p} f(p) dp$ if $F(p)$ is a continuous distribution with a frequency function $f(p)$, and $\sum \alpha_j e^{-\lambda p_j}$ if p follows a discrete distribution, taking the value p_j with relative frequency α_j. The effect of variation in p is, broadly, to reduce the slope of the relationship between P and λ. To illustrate this, text-figure 1 shows the response curves for four different distributions of p, including the special case when p is constant, and four different methods of plotting the results. The mean value of p is denoted by $\bar{p}$.

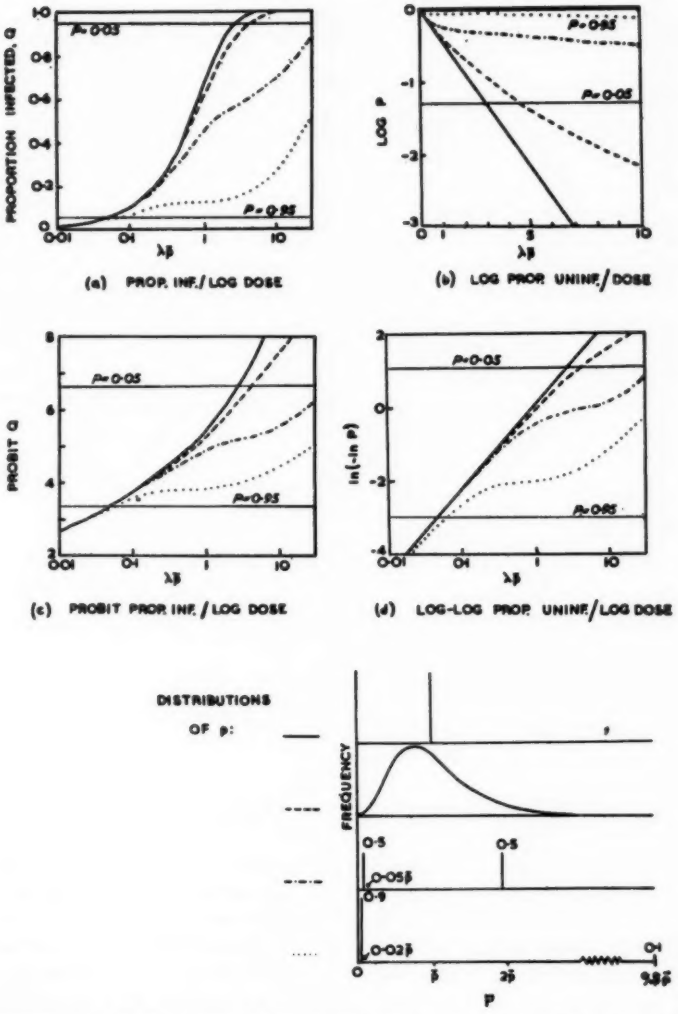
The distributions are:

i) p constant; P is given by (1).

ii) A Type-III (or Γ-) distribution with frequency function

$$f(p) \equiv F'(p) = 24 e^{-4p/\bar{p}} (4p/\bar{p})^3/\bar{p}.$$

This is the distribution of a variate proportional to χ^2 on 8 degrees of freedom. Strictly this is inadmissible for a quantity restricted between 0 and 1, but if $\bar{p}$ is sufficiently small, the theoretical curve will give only



TEXT-FIGURE 1.—Various ways of illustrating the response curve, for four different distributions of p.

a very small probability to values of p greater than 1, and the effect can be ignored. The coefficient of variation is 50 percent. The proportion of uninfected hosts is

$$P = (1 + \tfrac{1}{4}\lambda\bar{p})^{-4}$$

[*cf.* Armitage and Spicer (*3*), equation (17)].

iii) A "two-point" or "mixed-binomial" distribution, with p taking the values $0.05\bar{p}$ and $1.95\bar{p}$, each with probability $\frac{1}{2}$. Thus, there are

two types of host in equal proportions; one with a susceptibility (as measured by p) 39 times that of the other. For this distribution

$$P = \tfrac{1}{2}\{e^{-0.05\lambda\bar{p}} + e^{-1.95\lambda\bar{p}}\}.$$

iv) A two-point distribution so that p takes values $0.02\bar{p}$ and $9.8\bar{p}$ with probabilities 0.9 and 0.1, respectively.
Here

$$P = 0.9e^{-0.02\lambda\bar{p}} + 0.1e^{-9.8\lambda\bar{p}}.$$

The four methods of plotting are each used from time to time with this type of data. Since P is a function of $\lambda\bar{p}$, the abscissa in each graph of text-figure 1 has $\lambda\bar{p}$ on either an arithmetic or logarithmic scale. In practice, $\bar{p}$ would be unknown, and usually only the relative magnitudes of the λ's would be known. If the abscissa is the volume, or dilution, of a standard preparation used at each inoculum, a shift to left or right must be made in text-figure 1 (a), (c), and (d), and a change of scale in text-figure 1(b).

At low doses (small $\lambda\bar{p}$) the expression (3) becomes equivalent to

$$P \sim e^{-\lambda\bar{p}},$$

as may be seen from equation (3) of Armitage and Spicer (*3*) or equation (4) of the present paper. Hence, all the curves coincide at the left end.

For distribution (ii) a moderate degree of flattening becomes apparent at higher doses, as would be expected from equation (4) [4] of Armitage and Spicer, and from their figures 1 and 2. In distributions (iii) and (iv), p is given a higher coefficient of variation, and the departures from (1) are much more marked.

2. ESTIMATING THE DISTRIBUTION OF p

The problem of detecting a departure from the exponential response curve (1) has received a good deal of attention. Various significance tests are considered by Moran (*2*, *5*, *6*), Armitage and Spicer (*3*), Stevens (*7*), and Armitage (*8*). The problem of estimating some characteristic of the distribution of p has hardly been discussed. As Armitage and Spicer have pointed out, a small degree of variability of p is unlikely to be detected with the small number of replicate observations that is commonly used in many of these experiments, and any attempt to distinguish between various forms for $F(p)$ will require even more observations. Nevertheless, in some experiments, the number of host organisms at any dose level λ may be quite large, and the estimation problem may be worth considering.

If a particular functional form for $F(p)$ is postulated, the unknown parameters can be estimated by, for instance, the method of maximum

[4] In the right side of this equation, for p read $\bar{p}$.

likelihood. Moran (*2*) has discussed the maximum likelihood solution when $F(p)$ is assumed to be a Type-III distribution.

If the departure from the exponential curve is slight, it may be possible to make a rough estimate of the coefficient of variation of p by using the first-order approximation to p given by Armitage and Spicer's equation (4), which may be written

$$P \sim e^{-\lambda\bar{p}}\left\{1 + \frac{V}{2}(\lambda\bar{p})^2\right\}, \tag{4}$$

where $\sqrt{V}$ is the coefficient of variation of p. The equivalent expression using the log-log transformation is [5]

$$y \equiv \ln(-\ln P) \sim \ln(\lambda\bar{p}) - \frac{V}{2}(\lambda\bar{p}).$$

By fitting a straight line to the left end of the curve, the values of $\lambda\bar{p}$ at the lower (and hence also at the higher) doses can be estimated, and an estimate of V obtained from the departures from the line at the right end.

An alternative method of estimating V is given by Armitage and Spicer [(*3*), p. 410]. Moran's statistic T provides an estimate of the variance of $\log p$, which is approximately equal to $V(\log e)^2$ if V is not too large.

In a number of sets of data obtained from such experiments as those described later, in which animal tumors are initiated by viruses, it has been possible to obtain good fits by assuming a two-point distribution for p. If the high value of p bears a sufficiently high ratio to the low value, it is possible to get a rapid empirical fit to the response curve by the following considerations. Suppose p assumes the values p_1 and $p_2(>p_1)$ with probabilities α and $1 - \alpha$, respectively. Then

$$P = \alpha e^{-\lambda p_1} + (1 - \alpha)e^{-\lambda p_2}. \tag{5}$$

In practice, only the ratios of the λ's will be known, so let us write, for the ith dilution, $\lambda_i = \lambda_0 x_i$, where λ_0 is the number of particles in the undiluted preparation, and x_i is the dilution factor. For small values of λ_i,

$$P_i \sim e^{-\lambda_i\bar{p}} \text{ and } y_i \equiv \ln(-\ln P_i) \sim \ln(\lambda_i\bar{p}) = \ln(\lambda_0\bar{p}x_i). \tag{6}$$

For high values of λ_i,

$$P_i \sim \alpha e^{-\lambda_i p_1} \text{ and } y_i \sim \ln(\lambda_i p_1) = \ln(\lambda_0 p_1 x_i). \tag{7}$$

If p_2/p_1 is sufficiently great, the curve relating y to λ has a "shelf," *i.e.*, a point of inflection, with a very low gradient when, approximately, $y = \ln(-\ln \alpha)$.

If empirical estimates are made of α (from the position of the "shelf") and of $\lambda_0 p_1$ and $\lambda_0\bar{p}$ from the positions of the two asymptotes, these will provide the required specification of the two-point distribution—$\lambda_0 p_2$ is determined by a knowledge of $\lambda_0 p_1$, $\lambda_0\bar{p}$, and α. It should be emphasized

[5] Throughout this paper ln and log refer to logarithms to base e and 10, respectively. The symbol $\sim$ means "approximately equal to."

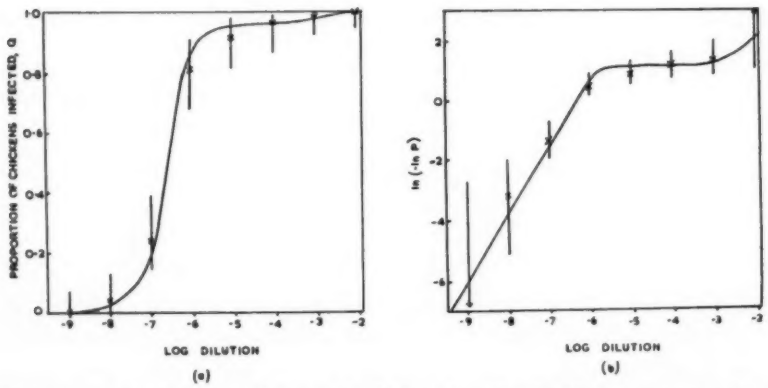
that in the absence of any direct counting method for evaluating λ_0, *e.g.*, by electron microscopy, we may hope to say something about the distribution of the product $\lambda_0 p$ but not about that of p alone. The procedure will be illustrated in the data discussed in the following sections.

3. AN EXPERIMENT WITH ROUS SARCOMA VIRUS

The data shown in table 1 are taken from table 7 of Bryan (*9*). Various dilutions of a preparation of the Rous sarcoma virus were inoculated into groups of New Hampshire Red chickens. Each chicken was inoculated at one site, in the subcutaneous tissue of the left wing web. The shape of the response curve, illustrated in text-figure 2, is typical of that expected from a two-point distribution of p, and an empirical fit may be obtained fairly rapidly by the procedure described in § 2.

TABLE 1.—(*See* text-fig. 2.) Incidence of tumors in chickens inoculated with Rous sarcoma virus [Bryan (*9*), table 7]

Log dilution	Total No. of chickens	Number with tumors	Proportion infected
−9	49	0	0
−8	50	2	.040
−7	49	12	.245
−6	49	40	.816
−5	50	46	.920
−4	50	48	.960
−3	50	49	.980
−2	50	50	1.000



TEXT-FIGURE 2.—Incidence of tumors in chickens inoculated with Rous sarcoma virus [Bryan (*9*), table 7]. The fitted response curve corresponds to a two-point distribution of p.

The vertical lines shown in text-figure 2 represent 95 percent confidence intervals for the responses at the different dilutions. These were obtained

first for the binomial responses shown in text-figure 2(a), and the log-log transforms of the limits were then inserted in text-figure 2(b).

The "shelf" appears to occur when y is in the region of 1, and as a suitable "round number" the estimate of α was taken to be 0.05 (for which $y \equiv \ln(-\ln\alpha) = 1.10$).

Since the log dilution is to base 10, the expected slope of the two asymptotes will be $\ln 10 = 2.3026$. A line of this slope fitted to the lower asymptote has been placed through the point ($\log x = -7$, $y = -1.6$), x being the dilution. From (6), this gives:

$$-1.6 \sim (2.3026)\,\{\log(\lambda_0\bar{p}) - 7\},$$

and as an estimate of $\log(\lambda_0\bar{p})$ we have

$$\log(\lambda_0\bar{p}) \sim 6.3.$$

The upper asymptote is much more difficult to estimate precisely. Its position could be placed almost as far to the right as one wished, *i.e.*, p_1 chosen as low as one wished, and still be reasonably consistent with the data. There is, of course, a limit to the extent to which the position could reasonably be moved to the left. A reasonable choice seemed to be to place the asymptote through the point ($\log x = -2$, $y = 1.4$). This would give

$$1.4 \sim (2.3026)\,\{\log(\lambda_0 p_1) - 2\}$$

and

$$\log(\lambda_0 p_1) \sim 2.6.$$

We estimate $p_1/\bar{p} \sim \text{antilog}\,(2.6 - 6.3) = 10^{-3.7} \sim 1/5000$.

Now $p_2 = (\bar{p} - \alpha p_1)/(1-\alpha)$, and α and $p_1/\bar{p}$ are sufficiently small for us to take $p_2 \sim \bar{p}$ to the degree of approximation used here.

Finally, then, we have

$$\lambda_0 p_1 \sim 10^{2.6} \text{ and } \lambda_0 p_2 \sim 10^{6.3}.$$

The fitted curve is

$$P = (0.05)\exp\{-10^{2.6}x\} + (0.95)\exp\{-10^{6.3}x\},$$

and is shown in text-figure 2.

More efficient estimates, with estimates of their sampling errors, could, of course, be obtained from a maximum likelihood solution. The purpose of this discussion is merely to show that some sets of data *can* be fitted by a two-point distribution, and that a reasonably good empirical fit can be made fairly quickly. This could be taken as the first approximation in an efficient iterative solution.

The fact that this particular set of data suggested that a small proportion of chickens was much more resistant than the rest was recognized by Bryan. In another paper, Bryan (*10*) describes briefly two experiments with White Leghorn chicks inoculated with the Rous sarcoma virus.

There are two strains differing widely in susceptibility, and one experiment was performed with each of the two strains. The results suggest that each strain contains a small proportion of chickens with a susceptibility characteristic of the other strain.

4. EXPERIMENTS WITH SHOPE PAPILLOMA VIRUS PROTEIN

Some experiments may provide further data on the distribution of p by a natural subdivision of the hosts into strata, in such a way that the variability of p is greater between strata than within.

The data shown in tables 2 and 3 have been provided by Dr. Ray Bryan. Experiment 1, the results of which are shown in table 2, is the experiment 1 summarized in table 1 of Bryan and Beard (*11*). The details are shown also in chart 1 of Bryan and Beard (*12*), but this chart contains certain errors, as may be seen by comparison with our table 2. Experiments 2a and 2b (our table 3) together comprise experiment 2 of Bryan and Beard (*11*); the details have not been published previously.

TABLE 2.—Occurrence of warts on different sites of rabbits inoculated with Shope papilloma virus protein—experiment 1 [Bryan and Beard, table 1 (*11*)—details from Bryan and Beard (*12*), with corrections]

		Log dilution:												
Rabbit	Side	−9.5	−9.2	−8.9	−8.6	−8.3	−8.0	−7.7	−7.4	−7.1	−6.8	−6.5	u*	R†
23	L	0	0	0	0	0	0	+	0	0	0	+	1	4
	R	0	0	0	0	0	0	0	+	0	0	+	1	3
22	L	0	0	0	0	0	+	+	+	+	+	+	5	0
	R	0	0	0	0	0	0	+	+	+	+	+	4	0
21	L	0	0	0	0	0	+	0	+	+	+	+	4	2
	R	0	0	+	0	0	+	+	+	+	+	+	6	3
16	L	0	0	0	0	0	+	+	+	+	+	+	5	0
	R	0	0	0	0	0	+	+	+	+	+	+	5	0
24	L	0	0	0	0	+	0	+	+	+	+	+	5	2
	R	0	0	0	0	0	+	+	+	+	+	+	5	0
19	L	0	0	0	0	+	0	+	+	0	+	+	4	5
	R	0	0	0	0	+	+	+	+	+	+	+	6	0
18	L	0	0	0	+	+	+	+	+	+	+	+	7	0
	R	0	0	+	0	0	+	+	+	+	+	+	6	3
20	L	0	0	0	0	+	+	+	+	+	+	+	6	0
	R	0	0	0	+	+	+	+	+	+	+	+	7	0
15	L	0	0	0	0	0	+	+	+	+	+	+	5	0
	R	0	0	+	+	+	+	+	+	+	+	+	8	0
13	L	0	0	0	+	+	+	+	+	+	+	+	7	0
	R	0	0	0	+	+	+	+	+	+	+	+	7	0
14	L	0	0	0	0	+	+	0	+	+	0	+	4	6
	R	+	+	+	0	+	+	+	+	+	+	+	9	4
17	L	0	+	+	0	+	+	+	+	+	+	+	8	3
	R	+	+	+	+	+	+	+	+	+	+	+	10	0
Positive sites		2	3	6	6	13	19	21	23	21	21	24		
Positive sites excluding rabbit 23:		2	3	6	6	13	19	20	22	21	21	22		

*u = Number of positives beyond log dilution −6.5.
†R = Stevens's range statistic.

TABLE 3.—Occurrence of warts on different sites of rabbits inoculated with Shope papilloma virus protein—experiments 2a and 2b [Bryan and Beard (*11*), table 2—details unpublished]

		Log dilution:											
Rabbit	Side	−9.5	−9.2	−8.9	−8.6	−8.3	−8.0	−7.7	−7.4	−7.1	−6.8	−6.5	u*	R†
Experiment 2a														
33	L	0	0	0	+	0	+	+	+	+	+	+	6	2
	R	0	0	0	0	+	+	+	+	+	+	+	6	0
34	L	0	0	+	0	+	+	+	+	+	+	+	7	2
	R	0	0	0	0	+	+	+	+	+	+	+	6	0
35	L	0	0	0	0	0	+	+	+	+	+	+	5	0
	R	0	+	0	0	+	0	+	+	+	+	+	6	5
36	L	0	0	0	+	+	+	+	+	+	+	+	7	0
	R	0	0	0	+	+	+	+	+	+	+	+	7	0
37	L	0	0	0	0	+	0	+	+	+	+	+	5	2
	R	0	0	0	0	+	+	0	+	0	+	+	4	5
Experiment 2b														
38	L	0	0	0	0	0	0	+	+	+	+	+	4	0
	R	0	0	0	0	0	+	+	+	0	+	+	4	4
39	L	0	0	0	0	0	+	+	+	+	+	+	5	0
	R	0	0	0	0	0	+	0	0	+	+	+	3	3
40	L	0	0	0	0	0	+	+	+	+	+	+	5	0
	R	0	0	0	0	0	+	+	+	+	+	+	5	0
41	L	0	0	0	+	0	+	+	+	+	+	+	6	2
	R	0	0	0	0	+	+	0	+	+	+	+	5	3
42	L	0	0	0	+	0	+	+	+	+	+	+	6	2
	R	0	0	0	+	+	+	+	+	+	+	+	7	0
43	L	0	0	0	0	0	+	+	+	+	+	+	5	0
	R	0	0	0	0	0	+	+	+	+	+	+	5	0
Positive sites		0	1	1	6	10	19	19	21	20	22	22		

*u = Number of positives beyond log dilution −6.5.
†R = Stevens's range statistic.

In these experiments, various dilutions of a preparation of the Shope papilloma virus protein are inoculated into sites of rabbits, and the incidence of warts is observed. Each rabbit receives two inoculations of each dilution, one on each side of the body. The sites are small areas along the ventral and ventrolateral surfaces of the body. According to Bryan and Beard (*11*), ". . . the sites inoculated with a given [dilution] were varied from animal to animal to cancel possible effects related to variations in the response of skin in different regions of the body." We shall assume that the dilutions were allocated at random to different sites, although this may not have been strictly so.

The log dilution shown in the present tables 2 and 3 (with a positive sign) is denoted by Bryan and Beard by the symbol pI, and represents the logarithm of the reciprocal of the number of grams of protein per inoculum. This is not strictly the log dilution of the original preparation, but differs from it only by a constant, and it seemed preferable to use the pI values for our log x, rather than to introduce a new set of numbers. In addition to the dilutions shown in tables 2 and 3, sites were inoculated with log dilutions −5.0 to −6.2 (in twofold steps), and all gave positive responses.

One feature of this experimental system should be mentioned at this stage. After a certain period has elapsed after inoculation with the virus, an animal may develop a resistance toward the infection, which manifests itself in a slowing down of the growth, or even complete retrogression, of the warts. If the one-hit model is at all applicable, then p must be taken to be the probability that a particle inoculated at a particular site initiates infection resulting in a wart, which is observed before any possible retrogression takes place. We shall discuss this matter again in § 5b.

The first question is whether the detailed results shown in tables 2 and 3 provide any evidence of variability in p *between sites on any one side of a rabbit*. Each of the 46 sides on the 23 rabbits provides a complete series of positive and negative responses, with one observation at each of a series of twofold dilutions. All dilutions before 10 give positive responses, and we shall assume that all dilutions beyond $10^{-0.5}$ give negative responses.

Of the tests that have been proposed for the detection of variability in p, Moran's (*2*) T test is inapplicable when the number of observations per dilution is 1. The most appropriate simple test appears to be that based on Stevens's (*7*) range R—which is also Moran's (*6*) $D + 1$. R is defined as the number of dilutions between (and including) the first at which not all observations are positive and the last at which not all are negative. Thus, for the left side of rabbit 23 in table 2, $R = 4$. The use of R is intuitively appealing, because a flattening of the response curve, such as we find with variability in p, will increase the chances of a positive response at a relatively low dose and of a negative at a relatively high dose, and consequently make it more likely that a large value of R is observed. The probability of observing various values of R, on the hypothesis that there is no variability in p, has been worked out, and we can see whether high values of R occur more frequently than might reasonably be expected on this hypothesis. The probability distribution of R for $n = 1$ is given in Stevens's (*7*) table 3, and in table 4 of the present paper we compare the observed and theoretical distributions. The agreement is very good, and we conclude provisionally that

TABLE 4.—Distribution of Stevens's R (=Moran's $D+1$)* for experiments 1, 2a, and 2b

R	Number of rabbit sides: Expt. 1	Expt. 2a	Expt. 2b	Total	Expected (Stevens)
0	14	5	7	26	23.00
1	—	—	—	—	—
2	2	3	2	7	8.42
3	4	0	2	6	6.17
4	2	0	1	3	3.85
5	1	2	0	3	2.18
6	1	0	0	1	2.37 ($R \geq 6$)
	24	10	12	46	45.99

*Stevens (*7*); Moran (*6*).

there is no evidence of variability in p from one site to another on the same side of the rabbit.

The next question is whether p varies from one rabbit side to another. Tables 2 and 3 show, for each side of a rabbit, u defined as the number of positive responses at dilutions beyond $10^{-6.5}$. We can regard u as a statistic linearly related both to the Spearman-Kärber estimate of the location parameter of the tolerance distribution [Armitage and Spicer (*3*)] and to the estimate of the density of effective particles proposed by Fisher (*13*), the calculation of which is facilitated by use of table VIII 2 of Fisher and Yates (*14*). The Fisher-Yates estimate of the log of the expected number of effective particles (λp in our notation) at the dilution $10^{-6.8}$ is, in fact

$$(0.301)u - K,$$

where K is obtained from Fisher and Yates's table VIII 2 and is very nearly constant. This estimate is highly efficient when n is large.

It is apparent from table 2 that rabbit #23 (for which $u = 1$ on both sides) differs appreciably in susceptibility from the rest. This may be confirmed by analyses similar to those to be described, and it will suffice to say here that this rabbit provides the main source of variability. Rabbit 23 exhibited relatively early retrogression, and this may well be the main reason why it appears to have such a low value of p. No other rabbits or sides of rabbits are particularly aberrant, so we proceed by omitting rabbit 23 and examining the remaining data more closely.

Table 5 provides an analysis of variance of u. The analysis in the upper part of the table shows that the random variation in u is significantly greater between than within rabbits, but provides no evidence of any systematic change in the mean value of u between the three experiments, or between the left and right sides of the rabbits, or of an interaction between these two factors. In the lower half of table 5, the variation between rabbits and within rabbits has been pooled, and again a significant variance ratio is found. This means that the two

TABLE 5.—Analysis of variance of u for experiments 1 (excluding rabbit 23), 2a, and 2b

	Degrees of freedom	Sum of squares	Mean square	Variance ratio	
Between rabbits					
Experiments	2	8. 873	4. 436		1. 66
Residual	19	50. 854	2. 677	2. 44*	1. 00
Within rabbits					
Sides	1	2. 273	2. 273	2. 07	
Sides × experiments	2	5. 842	2. 921	2. 66	
Residual	19	20. 885	1. 099	1. 00	
Total between rabbits	21	59. 727	2. 844	2. 16*	
Total within rabbits	22	29. 000	1. 318	1. 00	
	43	88. 727			

*$0.01 < P < 0.05$.

values of u on the same rabbit are correlated to an extent greater than can easily be explained by chance. The finding is quite independent of any assumption about the detailed nature of the dose-response curve, and means that the position of the curve is influenced by some factor varying from one rabbit to another. Whether this factor is a biological characteristic of the rabbit or whether it is some aspect of the environment experienced by the rabbit cannot, of course, be determined from data of this type.

The estimate of variance of u within rabbits is 1.318. Fisher (*13*) showed that on the theory of an exponential response curve, with an a-fold dilution series and n observations at each dilution, the theoretical sampling variance of u is $\log 2/(n \log a)$, or 1 when $n=1$ as here. (The result is true only when an infinite series of dilutions are used, and there is also a very small correction term, depending on the positioning of the dilutions in relation to the response curve.) The sum of squares within rabbits can therefore be regarded as a X^2 variate on 22 degrees of freedom, and is clearly not significantly high. This suggests that any variability in p must now be attributed to differences from one animal to another.

The component of variance of u, between animals, is estimated as

$$(2.844 - 1)/2 = 0.922,$$

since each animal provides two values of u. The estimate of variance of $\log p$ is therefore

$$(0.922)(\log 2)^2 = 0.0836.$$

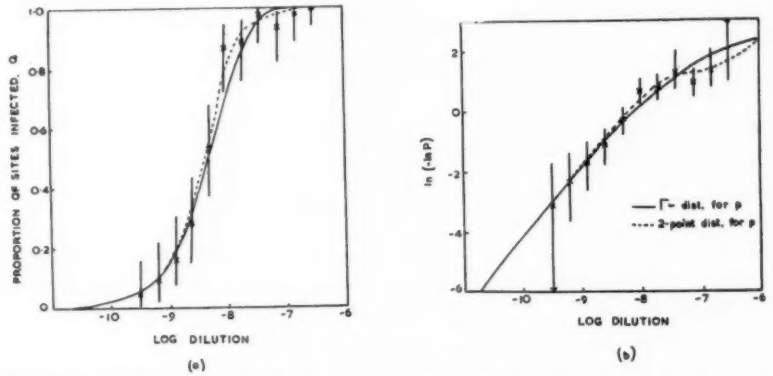
Let us now consider whether a distribution of $\log p$, with a variance equal to that estimated previously, will result in a response curve with a reasonable fit to the observed data. Table 6 shows the total numbers and proportions of positive sites at each dilution. The proportions are shown in text-figure 3, which also includes the log-log transformation. The vertical lines again indicate 95 percent confidence intervals based on

TABLE 6.—(*See* text-fig. 3.) Incidence of warts on sites of rabbits inoculated with Shope papilloma virus protein

Log dilution	Number of sites with warts: Expt. 1* (of 22)	Expt. 2a (of 10)	Expt. 2b (of 12)	Total (of 44)	Proportion infected
−9.5	2	0	0	2	0.045
−9.2	3	1	0	4	.091
−8.9	6	1	0	7	.159
−8.6	6	3	3	12	.273
−8.3	13	8	2	23	.523
−8.0	19	8	11	38	.864
−7.7	20	9	10	39	.886
−7.4	22	10	11	43	.977
−7.1	21	9	11	41	.932
−6.8	21	10	12	43	.977
−6.5	22	10	12	44	1.000

*Excluding rabbit 23.

binomial theory. Since the 44 sites at any one dilution do not form a random sample from a single population, but occur in 22 blocks of 2, the binomial limits are not strictly applicable. The intervals shown are too narrow, but the error is probably small, and the intervals provide some guide as to the relative accuracies of the various points.



TEXT-FIGURE 3.—Incidence of warts on sites of rabbits inoculated with Shope papilloma virus protein [Bryan (*11*), tables 1 (excluding rabbit 23) and 2]. *Continuous line* shows the fitted response curve given by a Type-III (or Γ-) distribution for p; *dotted line* corresponds to a two-point distribution for p.

As a first attempt to provide a suitable distribution for p, we consider a Type-III distribution for which var (log p) = 0.0836. Changing to natural logarithms, we have

$$\text{var } (\ln p) = (0.0836)\ (2.3026)^2 = 0.443.$$

Interpolating for $1/\kappa_2 = 1/0.443 = 2.26$ in table I of Bartlett and Kendall (*15*), we find that this logarithmic variance is provided by the distribution of the natural logarithm of a variance estimate on $\nu = 5.4$ degrees of freedom. The square of the coefficient of variation, denoted by Armitage and Spicer by V, is $2/\nu = 0.37$, and [from Armitage and Spicer's (*17*)]

$$P = \{1 + 0.37\ \lambda\overline{p}\}^{-2.7}. \tag{8}$$

Values of P (and hence y) were calculated for various values of $\lambda\overline{p}$ between 0.01 and 100. A reasonable fit to the lower end of the log-log response curve (upper part of text-fig. 3) was obtained by moving the fitted curve from left to right. The position shown by the continuous line in text-figure 3 gives $y = -4.2$ at $\log x = -10$. Since $y = -4.2$ is given by (8) when $\log \lambda\overline{p} = -1.82$, our estimate of $\lambda_0\overline{p}$ (the mean number of effective particles at $x = 1$) is $10^{10-1.82} = 10^{8.18}$.

The fitted curve reproduces to some extent the lowered responses at the higher doses, but gives too low a response at $\log x = -8$ and too high a response at $\log x = -7.1$. A better-fitting curve will clearly

have to exhibit a greater degree of flattening in this region, and the sigmoid shape characteristic of the two-point distribution for p is strongly indicated.

The variance of $\log p$, in the two-point distribution with parameters p_1, p_2, and α, is $\alpha(1-\alpha)\{\log(p_2/p_1)\}^2$. From the position of the shelf we might estimate α to be 0.05 (as in the example of § 3), and equating var $(\log p)$ to 0.0836, we have

$$\log(p_2/p_1) = \sqrt{(0.0836/0.0475)} = 1.33,$$

and $p_2/p_1 = \text{antilog}(1.33) = 21$. With $\alpha = 0.05$, this ratio implies $p_1 = 0.05\,\bar{p}$ and $p_2 = 1.05\bar{p}$, and the fitted response curve is

$$P = 0.05 \exp(-0.05\,\lambda\bar{p}) + 0.95 \exp(-1.05\,\lambda\bar{p}).$$

From the lower part of the curve we make the same estimate of $\lambda_0\bar{p}$ as before, and the fitted curve is shown as a broken line in text-figure 3.

The fit is satisfactory, and is clearly an improvement on that provided by the Type-III distribution. There is unfortunately one consideration which prevents the acceptance of the two-point distribution as a suitable model for the variation in p in this experiment. This difficulty will be discussed in the next section.

5. SOME DIFFICULTIES IN INTERPRETATION OF PAPILLOMA DATA

a. The Two-Point Distribution

The analysis of experiments 1, 2a, and 2b presented in § 4 suggests that an acceptable model for the variability in p is that this is confined to variation between animals, and that p follows a two-point distribution with about 5 percent of animals having a value of p some 1/21 the value attributed to the remaining 95 percent. It is difficult to see how this sort of distribution would be suggested by the 22 animals considered here, unless one or perhaps two animals (*i.e.*, about 5% of the total) were clearly more resistant than the rest. Since $21 = 2^{4.4}$, we should expect the mean value of u for this small minority group to be about 4 to 5 units less than the mean for the remainder.

Each animal shown in tables 2 and 3 contributes two values of u, and their mean value $\bar{u}$ is (apart from a constant) an estimate of the value of $\log_2 p$ characteristic of this animal. The distribution of $\bar{u}$ is shown in table 7. (The omitted rabbit 23 has $\bar{u} = 1$.) The distribution is perhaps bimodal, but there is no suggestion that one or two rabbits have a value of $\bar{u}$ some 4 or 5 units *less* than the rest. There is one rabbit (#17) with a particularly *high* value of $\bar{u}$ (= 9.0), and hence a high value of p. It is not surprising that this has no noticeable effect on the response curve, as a small proportion (about 5%) of hosts with a high value of

p will produce a "shelf" on the log-log plot at low values of y (*i.e.*, $\ln(-\ln 0.95) = -3.0$), which could hardly be detected with the number of observations made here.

TABLE 7.—Distribution of values of a for the rabbits in experiments 1 (excluding rabbit 23), 2a, and 2b

a	Number of rabbits: Expt. 1*	Expt. 2a	Expt. 2b	Total
4.0	0	0	2	2
4.5	1	1	0	2
5.0	4	0	2	6
5.5	0	1	1	2
6.0	0	1	0	1
6.5	4	1	1	6
7.0	1	1	0	2
7.5	0	0	0	0
8.0	0	0	0	0
8.5	0	0	0	0
9.0	1	0	0	1
	11	5	6	22

*Excluding rabbit 23 (for which $a = 1$).

The most likely explanation of the paradox is that there may be some variability in p *within animals*, which could be detected by a more sensitive test than that used in table 4. Armitage (*8*) has described a test, efficient for large n, based on the derivative of the log likelihood with respect to a parameter representing small degrees of variation in p. By enumerating all possible combinations of results for $n = 1$, it can be shown that this test is almost equivalent not to the Stevens-Moran test, but to another test of equal simplicity. The statistic to be calculated, J, is the number of positive results at dilutions beyond that at which the first negative occurs. (The definition refers only to the case $n = 1$[6].) For the left side of rabbit 23, for instance, the first negative occurs at $\log x = -6.8$, and there is one positive at a higher dilution ($\log x = -7.7$); hence $J = 1$.

The observed distribution of J, together with the expected frequencies, is shown in table 8. There is now some suggestion of an excessive frequency for $J = 3$ or 4, although the numbers are small. Four of these five sides with $J = 3$ or 4 constitute a negative result at $\log x = -6.8$ or -7.1, and are largely responsible for the flattened slope at these low dilutions.

[6] *Note* the asymmetry of the definition. It is easily seen that $J - \frac{1}{2}$ is the number of dilution steps between the first negative and the Spearman-Kärber estimate of the LD50. Thus J is particularly sensitive to the occurrence of negative results at surprisingly high doses, a feature which would be expected of a highly efficient test in view of the importance of this part of the response curve. A further discussion of the J-test will appear in a separate paper.

TABLE 8.—Distribution of the statistic J^* for experiments 1, 2a, and 2b

	Number of rabbit sides in:				
J	Expt. 1	Expt. 2a	Expt. 2b	Total	Expected
0	14	5	7	26	23.0
1	6	3	3	12	15.6
2	1	1	1	3	5.8
3	2	1	1	4	1.4
4	1	0	0	1	0.2
>4	0	0	0	0	0.0
	24	10	12	46	46.0

*J = number of positive responses after the first negative.

The safest conclusion about these experiments would perhaps be that there is some variability in p, barely detectable, between sites on a rabbit; and that there is clear evidence of heterogeneity between animals, 1 rabbit of 23 having particularly low susceptibility and another having particularly high susceptibility.

b. Effect of Retrogression

The phenomenon of retrogression has been described in § 4. We have assumed, essentially, that the particles act independently, not only in the initiation of infection but also in the manifestation of a tumor. For a particular host organism let $p = p'\beta$, where p' is the probability that a particle is successfully infective, *i.e.*, initiates infection, and β is the probability that an infection, once started, produces a detectable tumor.

Denote by Θ_k the probability that a host organism which has received exactly k successfully infective particles shows a detectable tumor. Then it is easily seen that

$$\Theta_1 = \beta, \Theta_2 = 1 - (1 - \beta)^2,$$

and, in general,

$$\Theta_k = 1 - (1 - \beta)^k, \tag{9}$$

which approaches unity as $k \to \infty$.

It may be, however, that the probability of manifestation, Θ_k, depends on k in some other way than that indicated by (9). If, for instance, manifestation depends largely on whether the virus population has reached a certain total size before retrogression occurs, it may well be that Θ_k is small when k is less than some value k_0, rises rapidly in the neighborhood of k_0, and remains near unity for $k > k_0$. In the absence of host variability, the response curve will then resemble that appropriate to a multi-hit curve, the number of hits being k_0. Such a curve is known to be *steeper* than the exponential. Host variability in either p' or k_0 would tend to flatten the response curve. In the data examined here there is, of course, no evidence of a steeper curve than the exponential,

but it is possible that retrogression, having the opposite effect to host variability, masks some of the variability which is really present.

c. Response Times

No attempt is made in this paper to provide a theoretical explanation of the distributions of response time—the time between inoculation and the appearance of the tumor. This time is regarded by Iversen and Arley (*16*) as the sum of the *excitation time*—the time between inoculation and the event which starts the tumor—and the *growth time* of the tumor. It would be expected that the excitation time, if not also the growth time, would tend to decrease with increasing virus inoculum, but the exact nature of the relationship would depend on the postulated mechanism of tumor induction. The distributions published by Bryan and Beard (*12*) show the expected negative correlation.

Meynell and Meynell (*17*) point out that on the one-hit theory of infection the mean response time would tend to approach an upper limit as the dose decreases below the LD50; for most of the positive responses at these low doses will be the result of infection by only one effective particle, and the mean response time for these one-particle infections will be constant. There is no suggestion of this tendency in the response-time distributions of Bryan and Beard (*12*), but the number of positive results at low doses is necessarily small.

Iversen and Arley (*16*) postulate that tumor induction takes place by excitation of a "cancer control centre," the probability of such an event per unit time being proportional to the concentration of virus particles at a given instant. This concept is in principle compatible with a one-hit theory of infection, but Iversen and Arley use a nonrandom theory of infection, whereby each virus inoculum, however small, is assumed to grow exponentially. This appears to ignore the particulate nature of the inoculum, and has the consequence that each inoculum, however small, will ultimately produce a tumor.

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Peripheral Blood Changes and Liver Catalase Response to Isologously Transplanted Rat Leukemia IRC 741 [1,2]

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SUMMARY

The effects of the growth of acute leukemia IRC 741 were observed in Fischer line 344 male rats. Erythrocyte counts and hemoglobin value dropped significantly during the terminal stages. There was a progressive significant increase in leukocyte level during the development of the disease, the terminal counts being 20 times higher than normal. Blood glucose values decreased throughout the course of the disease, and amino acid values remained relatively unchanged until the terminal stage when a significant increase was observed. Liver catalase activity values were elevated slightly through day 11. This was followed by a marked reduction at day 15 and a more pronounced decrease at the terminal stage. Liver weight per body weight ratios remained relatively unchanged during the disease. Spleen weight per body weight ratios fluctuated during the early stages of the disease but increased steadily toward the terminal stage.—J. Nat. Cancer Inst. 23: 1331–1338, 1959.

THE EFFECTS of malignant disease on various components of the blood and liver catalase have been the subject of intense investigation by numerous workers. However, relatively little investigation has been done on the Fischer line 344 rat and the transplanted neoplasm IRC 741.

Dunning and Curtis (*1*) first described this neoplasm as an acute leukemia that occurred spontaneously in a rat bearing the transplanted acetylaminofluorene-induced mammary adenocarcinoma IRC 741. Reich and Dunning (*2*) and Dunning and Reich (*3*) investigated erythrocyte and leukocyte counts and hemoglobin values in various strains of rats after inoculations with several different tumors and observed a decrease in numbers of erythrocytes, a significant decrease in hemoglobin, and a very rapid increase in the leukocyte counts in the advanced stages. Dunning and Curtis (1) obtained similar results with IRC 741. Elevated amino acid values have been observed in leukemic patients (*4–6*) and in tumor-bearing mice and rats (*7–9*).

[1] Received for publication May 27, 1959.

[2] Included in this study is the abstract of a thesis submitted by the senior author to the Graduate School of the University of Miami, in partial fulfillment of the requirements for the degree of Master of Science.

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In 1916, Brahn first observed decreased liver catalase activity values in humans with malignant growths. Depressed liver catalase activity has been observed, by others, in tumor-bearing rats (*10–15*) and mice (*16, 17*). Liver enlargements in rats with growing tumors have been repeatedly observed in several laboratories (*10, 18–20*). Splenomegaly was observed in tumor-bearing rats by Appleman *et al.* (*20*), Stewart and Begg (*21*), and Antopol *et al.* (*22*).

In the present investigation, erythrocyte, leukocyte, hemoglobin, blood glucose, and amino acid determinations were made on the blood of Fischer line 344 rats during the course of the development of transplanted acute leukemia IRC 741. Liver catalase activity was determined both in normal rats and in rats inoculated with IRC 741 at various intervals. Additional data on changes in liver and spleen weight were also obtained during the course of the disease.

MATERIALS AND METHODS

Fischer line 344 male rats, varying in age from 75 to 150 days and in weight from 130 to 250 gm., were maintained at room temperature and fed on Purina laboratory chow supplemented occasionally with fresh lettuce leaves. All rats, under light anesthesia, received subcutaneous injections, in the right side, of approximately 2 $mm.^3$ of tissue from a subcutaneous tumor of IRC 741.

Blood determinations.—Rats to be bled were weighed, placed in cylindrical wire-mesh cages, and maintained in a warming box at 30° C. for 15 to 30 minutes to promote general vasodilation. Peripheral blood samples were obtained by amputating a thin segment of the tail with sharp scissors and then gently stroking the tail with a small piece of cotton held between the thumb and forefinger. Blood samples were collected in glass capsules containing potassium oxalate. After one or two normal samples had been obtained, animals were inoculated with the tumor. Subsequent samples were taken on the 3d, 10th, and 15th to 17th day (terminal).

Immediately after blood samples were drawn, a deproteinized filtrate was made by the method of Somogyi (*23*). Blood glucose values were determined by the method of Loewus (*24*), with a Fisher electrophotometer. Amino acid values were obtained by the modified Folin method of Danielson (*25*). Erythrocyte and leukocyte counts were made by standard procedures. Hemoglobin values were determined by the method presented in the Fisher Electrophotometer Manual (*26*).

Catalase determination.—All animals were killed by etherization, and the livers removed, blotted on filter paper until superficially dry, and then weighed to the nearest milligram. Exactly 200 mg. of tissue was taken from the median right lateral lobe of the liver. This was ground in a Ten Broeck homogenizer in an ice bath with 10 cc. of distilled water, until the mixture was homegeneous. An additional 10 cc. of water was used to rinse the homogenizer and was added to the original homogenate. The

rest of the catalase determination was performed by the method of Euler and Josephson, as described by Ronkin (*27*). Varying amounts of homogenate were used, making the 12-minute titration value 4 to 9 cc. of 0.005 N $KMnO_4$. The titration values were plotted on semilogarithmic paper, the slope of the line was determined, and then by multiplying this fraction by the dilution, the units of activity per gram were obtained.

Inoculated animals were killed at 3- to 4-day intervals, and the catalase values determined. All terminal values were taken from rats used in the blood determinations. About one-third the terminal animals were killed when death appeared imminent; the remainder were allowed to die. In several instances when catalase determinations could not be made immediately, livers were frozen in a deepfreeze for several days. In most instances the spleen was removed, weighed, and the ratio of spleen weight to body weight and of liver weight to body weight was determined.

RESULTS

Blood Determinations

Table 1 shows the sample size, the mean, and the standard error for erythrocyte and leukocyte counts and hemoglobin values before inoculation of the rats and during the course of the development of the disease. A significant decrease in erythrocytes was observed at day 3, which was followed by an insignificant increase at day 10. Terminal values indicated a more pronounced decrease than at day 3 and were significantly lower than the means before inoculation and at day 10. The mean leukocyte count at day 3 had increased about 700 per mm.^{3} over the mean before inoculation. Values at day 10 were significantly higher than those before inoculation and at day 3. At the terminal stage, leukocyte counts had

TABLE 1.—Erythrocyte counts, leukocyte counts, and hemoglobin values in Fischer rats as normals and at various intervals after inoculation of IRC 741

	Days after inoculation:			
	0	3	10	15–17
Erythrocytes*				
Mean × 10^3	6,825	6,090	6,543	5,535
Number of observations	35	16	15	13
Standard error × 10^3	247	254	297	386
Leukocytes				
Mean	8, 260	8, 950	10, 470	161, 000
Number of observations	35	16	15	13
Standard error	470	620	527	14, 000
Hemoglobin				
Mean	13. 2	13. 4	12. 6	10. 9
Number of observations	39	16	15	13
Standard error	0. 2	0. 2	0. 2	0. 7

*Erythrocyte and leukocyte values are counts per mm.^{3} Hemoglobin values are grams of hemoglobin per 100 cc. of blood.

increased almost 20-fold when compared to the normal value and approximately 15-fold when compared to the values at day 10. The terminal counts ranged from 90,000 to 240,000. Hemoglobin values showed a slight, insignificant rise by the 3d day when compared to values prior to inoculation. Values at days 10 and 15 to 17 were significantly lower than those before inoculation and at day 3. During the day 10-terminal stage period there was a significant drop in hemoglobin, which was more pronounced than that during the period between the 3d and the 10th days.

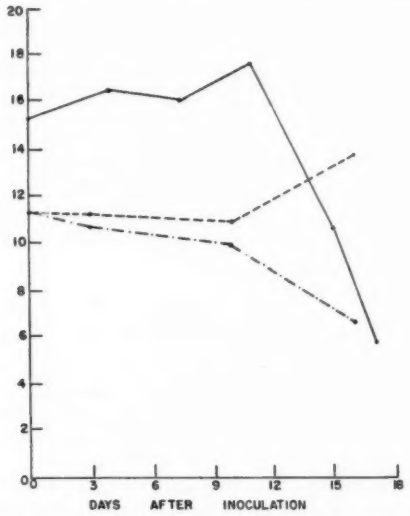
The blood glucose and amino acid values are presented in table 2 and text-figure 1. There was a pronounced decrease in blood glucose values after inoculation, the decrease being more pronounced toward the terminal stage. Blood glucose values in each interval were significantly lower than those during preceding intervals. Amino acid values indicated a slight decrease before inoculation to day 3 and to day 10, though differences were not significant. The terminal mean is significantly different from that during preceding intervals. Terminal readings showed an increase of 2.4 mg. per 100 cc. when compared to the normals, and an increase of 2.8 mg. per 100 cc. when compared to values at day 10.

TABLE 2.—Milligrams of blood glucose and amino acids per 100 cc. of blood in Fischer rats as normals and at various intervals after inoculation of IRC 741

	Days after inoculation:			
	0	3	10	15–17
Blood glucose				
Mean	113	107	99	66
Number of observations	38	15	15	13
Standard error	2	3	3	5
Amino acids				
Mean	11.3	11.2	10.9	13.7
Number of observations	32	16	15	13
Standard error	0.4	0.5	0.6	0.7

Catalase

Liver catalase activity values are presented in table 3 and text-figure 1. A slight though insignificant increase in catalase activity occurred through days 7 to 8. There was an increase in the values at day 11, which was significantly higher than the average before inoculation but not significantly different from values at days 3 to 4 and 7 to 8. Days 15 to 17 showed a significant decrease in activity from day 11. Some readings at day 15 were of the same magnitude and others were much lower than the ones obtained at earlier intervals, which indicated that a decrease was taking place or had already taken place. The mean at days 15 to 17 showed a 40 percent decrease from the average value at day 11. At the terminal stage the catalase activity had decreased approximately 60 percent when compared to the values before inoculation. At most intervals, and especially at day 11, the range of values was rather wide.



TEXT-FIGURE 1.—Blood glucose, amino acids, and catalase activity before inoculation and at various intervals after inoculation of IRC 741.
—·—: Milligrams of blood glucose per 100 cc. of blood × 10.
– – – –: Milligrams of amino acids per 100 cc. of blood.
————: Units of catalase activity per gm. of liver × 10^3.

TABLE 3.—Units of catalase activity per gram of liver in Fischer rats as normals and at various intervals after inoculation of IRC 741

	Days after inoculation:					
	0	3–4	7–8	11	15–17	Terminal
Mean	15, 330	16, 511	16, 112	17, 719	10, 619	5, 723
Number of observations	12	8	8	8	8	15
Standard error	1, 041	1, 271	878	812	1, 628	259

Table 4 shows the relationships between body, liver, and spleen weights. Ratios obtained at days 3 to 4 and 7 to 8 were slightly higher than normal values but, in general, liver weight remained almost in the same proportion to body weight through day 11. There was a significant increase in the ratios at day 15, followed by a decrease at the terminal stage. Table 4 shows that spleen weight was higher at day 3 when compared to the normal values. Thus, average spleen weight decreased to a value slightly higher than normal at days 7 to 8. At intervals after days 7 to 8, spleen weight increased progressively until it reached 3 times the normal value at the terminal stage.

TABLE 4.—Organ weight per body weight ratios of Fischer rats before inoculation and at various intervals after inoculation of IRC 741

	Days after inoculation:					
	0	3–4	7–8	11	15	Terminal
$\frac{\text{Liver weight}}{\text{Body weight}} \times 10^{-4}$						
Mean	418	442	428	399	490	451
Number of observations	13	8	8	8	8	15
Standard error	12	26	10	17	31	19
$\frac{\text{Spleen weight}}{\text{Body weight}} \times 10^{-4}$						
Mean	354	591	381	552	914	998
Number of observations	9	8	8	7	8	14
Standard error	66	86	74	92	105	63

DISCUSSION

In the present investigation the normal erythrocyte and leukocyte counts and hemoglobin values are slightly lower than, but not significantly different from, the results of other investigations (*1–3, 28*). Decrease of erythrocyte and hemoglobin values during the course of the disease, in this study, is of about the same magnitude as that reported by Dunning and Reich (*3*). Increases in leukocytes during the various stages of the disease, and especially toward the terminal stage, run almost parallel to those of Dunning and Curtis (*1*). It has been reported (*29*) that several investigators have shown lower blood glucose values as well as diminished urinary glucose in diabetic rats bearing a tumor. The blood glucose results (text-fig. 1) show a gradual decrease in blood glucose during the early period, with a greater decrease in the terminal stages. An absolute comparison cannot be made with the results in the literature because some of the reported data are on urinary glucose or on fasted rats. The increase in terminal amino acid values (text-fig. 1) conform generally to the observations of Sassenrath and Greenberg (*9*). Elevated terminal amino acid values have been reported in leukemic patients (*4, 6*).

The normal liver catalase activity values (table 3, text-fig. 1) obtained in this study are comparable to the values reported by other investigations (*14, 20*). The values during the course of the disease also are comparable to the results of Appleman *et al.* (*20*) who observed a decrease. However, decrease in terminal catalase values observed in the present work was not as great as that reported by other workers (*12–15*), who noted a decrease of 75 to 95 percent.

In the present study, normal liver weight per body weight ratios (table 4) show relatively little change and confirm the findings of Dounce and Shanewise (*10*). However, others (*10, 19, 20*) have noted significant increases in liver weight in rats with malignant disease. Spleen weight, both before inoculation and during the course of the disease, in this study, corresponds to increases noted by Appleman *et al.* (*20*).

The relationships between hemoglobin and number of erythrocytes seem clear, since their observed values parallel each other and their decreased values can be attributed to anemia which becomes greater toward the terminal stages. The data also indicate relationships between the decrease in blood glucose and the increase in number of leukocytes. The decrease in blood glucose could be due to the malfunctioning of the liver in regulating the blood glucose level and to the increased number of leukocytes utilizing greater amounts of glucose.

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Effect of Formaldehyde Wound Wash on Development of Local Wound Recurrences [1]

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SUMMARY

A group of patients with primary operable cancer of the cervix uteri and another group of patients with head and neck cancers were treated by *en bloc* surgical excision, followed by a therapeutic wound wash of 0.5 percent formaldehyde. The survival and local recurrence experience of these groups of patients were compared with that of comparable groups of patients who had similar surgery but no therapeutic wound wash. No significant difference in survival or rate of local recurrence could be demonstrated. Under the circumstances of this study, 0.5 percent formaldehyde was found to be of no value as a therapeutic wound wash.—J. Nat. Cancer Inst. 23: 1339–1345, 1959.

LOCAL REGROWTH of cancer in an operative wound after surgical excision of a cancer remains a frequent finding in treatment-failure cases. The finding of clumps and individual cancer cells in wound washings and the multicentric character of many local recurrent cancers have suggested that these recurrences could be due to a seeded wound. This has encouraged surgeons to attempt local chemotherapy of operative wounds. In the past, many different types of wound wash have been used, including saline, Dakin's solution, ether, alcohol, bichloride of mercury, and, more recently, Clorpactin. The effectiveness of these agents in preventing local recurrent cancer has not been reported. A more complete review of the subject of cancer wound implants has recently been published by Haverback and Smith (*1*).

Experimental work previously reported from this laboratory (*2*) demonstrated that formaldehyde was effective in preventing the implantation and growth of cancer cells in artificially seeded animal wounds. Formaldehyde (0.05%–0.1%) applied at times varying from 5 minutes before to 24 hours after tumor inoculation prevented growth of tumor in 80 to 85 percent of the animals. If some of the recurrent cancer nodules that develop in human operative wounds are caused by implantation and growth of the free cells demonstrated in wound washings, the effect

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

of a drug on growth of these implants would be similar to that seen in the animal model.

This study reports the results of using a wound wash of 0.5 percent formaldehyde on the development of local recurrent cancer and on survival in a group of patients treated by *en bloc* surgical excision for cancer of the cervix uteri and the head and neck region.

MATERIALS AND METHODS

A group of patients with primary operable cancer was treated by the usual *en bloc* resection of their primary tumor and adjacent lymph drainage area. After removal of the cancer and before closure of the wound, the entire operative area was sprayed with 200 cc. of saline and the washings collected for cytologic study. This was followed by a therapeutic wash of 200 cc. of 0.5 percent formaldehyde. The washings were applied by a fine, forceful spray and the wound was then closed in the usual manner without removing the excess drug.

In general, the patients included in this study had locally advanced cancer, but none had X-ray or clinical evidence of distant metastases. Well over 100 operative wounds of all types have been treated with formaldehyde. The patients with carcinoma of the cervix uteri and those with cancer arising in the mucous membrane of the head and neck areas comprise groups large enough to allow statistical study of the results of this wound treatment. The only cases excluded were those that did not have clear surgical margins; *i.e.*, those in which histologic study of the operative specimen revealed tumor extending to the line of excision. Exclusion of these cases was necessary, for topical chemotherapy could not be expected to destroy cancer embedded in tissue remaining in the wound edges after excision.

The treated patients with carcinoma of the cervix uteri and those with cancer of the head and neck regions were compared with similar groups of control patients. The patients in the control group received the same surgical treatment and had a saline wound wash for tumor-cell cytologic study, but did not have the therapeutic formaldehyde wound wash given the treated group. In previous studies of cancer-cell contamination of operative wounds (*3*), no therapeutic effect could be demonstrated by washing a wound with 200 cc. of saline. The group that had the formaldehyde wound wash was comprised of patients treated between January, 1956, and December, 1958. The control group was comprised of patients treated between September, 1953, and July, 1958. Since the control and treated groups were obtained over different periods, a comparison was made between groups to assure that the patients were comparable regarding type of surgery and extent of disease. The data are given in table 1. From these data it can be seen that the groups were as comparable as could be expected. A higher proportion of untreated

TABLE 1.—Type of surgery and extent of disease in treated and control groups

Site	Number of patients	Type of surgery			Extent of disease		
		Incontinuity resection of primary and radical neck dissection	Radical neck dissection only	Radical resection primary only	Number of cases with positive nodes	Number of cases with vascular invasion	Surgery margins clear of tumor
Head and neck							
Treated	31	21	4	6	19	6	All
Untreated	33	22	5	6	19	7	All
		Pelvic exenteration	Radical hysterectomy				
Cervix							
Treated	22	18	4		7	5	All
Untreated	23	17	6		12	4	All

patients with cancer of the cervix had positive nodes; however, if there is any bias to the comparison, it should be in favor of the treated group.

TOXICITY

Toxicity studies on rabbit and mouse brachial plexuses demonstrated that impairment of nerve function was related to the concentration of formaldehyde and the time it was allowed to be in contact with the nerve (*2*). Concentration of 0.5 percent over a period of 5 minutes was found safe. No impairment of wound healing was observed with concentrations 3 or 4 times that used in this study. In over 100 human operative wounds that have been washed with 0.5 percent formaldehyde, no serious toxic effect has been seen. In one instance after resection of a tongue, a portion of the mandible, and a radical neck dissection in continuity, a "chemical cellulitis" developed in 18 to 24 hours after surgery. This subsided spontaneously and required no treatment. When formaldehyde was sprayed directly on the carotid bulb and vagus nerve, occasional blood pressure changes were observed. In most instances a drop in blood pressure was noted. On occasion, a similar drop in blood pressure occurred when saline was forcefully sprayed on the vagus nerve. This hypotension was corrected or prevented by an injection of novocain into the carotid bulb or the facial covering of the vagus nerve. When the drug was sprayed into the pelvis after pelvic exenteration, a rise or fall in the blood pressure was occasionally seen. In no instance were blood pressure changes detrimental to the patient.

Concentrations of 1 percent and as high as 3.5 percent formaldehyde have been used without incident in the pelvis. A gauze sponge saturated with 3.5 percent formaldehyde is routinely sutured over fungating tumor surfaces; *i.e.*, a formaldehyde gauze pack placed in the vagina over a cervix tumor mass with a cuff of vagina turned to seal off the sloughing cancer surface. No toxicity has been observed under such circumstances.

RESULTS

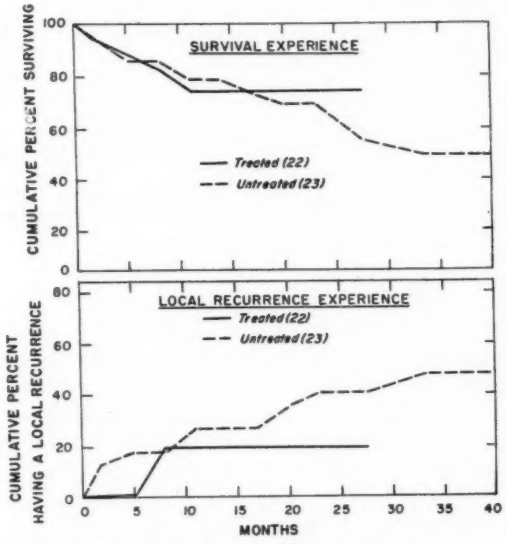
An effective local wound chemotherapy should decrease the incidence of local recurrent cancer in the area of surgery and increase the time of survival. This was not observed.

The data on local recurrence experience are given in table 2 and plotted in text-figures 1 and 2. Since the treated group has been observed over a shorter period, the groups have been adjusted for follow-up, with the use of a life-table analysis (*4*).

At 6 and 12 months, the calculated local recurrence rate in the treated cervix group is lower than in the controls, while in the head and neck group, the opposite is true, with the formaldehyde-treated group having a higher local recurrence rate. None of these differences are statistically

TABLE 2.—Local recurrence experience

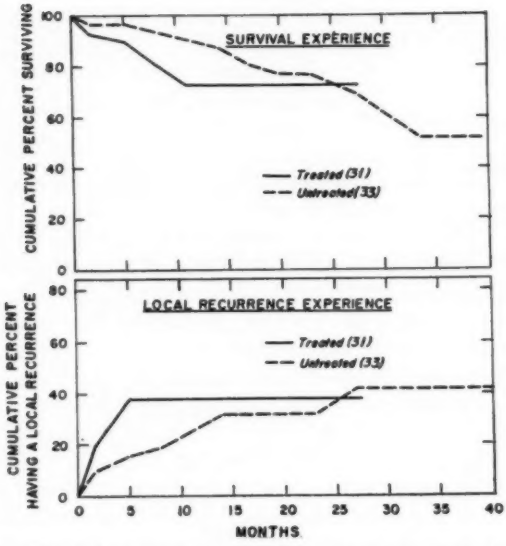
	Total proportion of local recurrences	Cumulative proportion of local recurrences adjusted for follow-up	
Cervix group		To 6 months	To 12 months
Formaldehyde treated	3 of 22 patients = 14%	0. 06	0. 20
Untreated	10 of 23 patients = 43%	0. 18	0. 27
Head and neck group			
Formaldehyde treated	11 of 31 patients = 35%	0. 38	0. 38
Untreated	12 of 33 patients = 36%	0. 16	0. 27



TEXT-FIGURE 1.—Treated *versus* untreated cases of cancer of the cervix: life-table analysis of survival experience and local recurrence experience.

significant. Previously reported studies of wound washings and local recurrent cancer have shown that 80 percent of local recurrences occurred within 12 months of surgery (*3*). In this study, all local recurrences to date in the combined treated groups occurred within the first 12 months after surgery (text-figs. 1 and 2). In the control groups, 14 of 22 patients developed local recurrences in the 1st year after surgery.

The survival experience of patients in the two groups was not significantly changed by the wound treatment. This is shown in text-figures 1 and 2. The cumulative percent surviving at 12 months is not significantly different in the treated and untreated groups.



TEXT-FIGURE 2.—Treated *versus* untreated cases of cancer of the head and neck: life-table analysis of survival experience and local recurrence experience.

DISCUSSION

Clinical experience with individual cases, as well as this study of groups of cases, demonstrates that local wound treatment with 0.5 percent formaldehyde as applied in this study did not influence to any measurable extent the survival or local recurrence experience. This disappointing ineffectiveness of local formaldehyde treatment can possibly be explained on the basis of lack of contact of the drug with the cancer-cell implants. It is possible that the drug did not come in contact with all such free cells. Transplantation of clumps of cells by suture thread to deep layers does occur and gives rise to recurrence associated with suture material, frequently seen by the pathologist (*1*). These buried implants would not come in contact with the topical chemotherapy.

An additional factor that possibly may be responsible for this lack of effect of formaldehyde treatment is a "reseeding" of the wound by tumor cells from local blood. Small amounts of blood, lymph, and tissue juices continue to drain into these wounds for 48 to 72 hours after surgery. Tumor cells have been demonstrated in this drainage (*5*).

In local chemotherapy studies with transplantable animal tumors, accentuation of tumor growth in "artificially seeded wounds" has been demonstrated. When formaldehyde or Clorpactin was placed in an animal wound before the tumor inoculum, or when the drug was diluted below a certain concentration, an increase in the amount of tumor growth

and a decreased latent period of growth were observed (*2*, *6*). Both factors that were associated with accentuation of growth in the animal studies could theoretically be present in the clinical circumstances that existed in this study. These operative wounds could be "reseeded" by tumor cells from lymph and blood that accumulate in wounds after excisional cancer surgery. The data presented in text-figures 1 and 2 do not demonstrate a pretreatment accentuation effect. The local recurrence experience in the head and neck group was slightly higher at the 6- to 12-month follow-up period in the treated group than in the control group. In the cervix group, an opposite effect was seen. These differences are not statistically significant.

Further maturation of this data, especially in the treated groups in which about one half of the patients have been followed more than 12 months, may alter the survival and local recurrence experience. However, it could not improve the results to a significant degree, for the experience to date in the two groups is nearly the same. It would appear, therefore, that if local chemotherapy of operative wounds is to be effective, modification of drug dosage, types of wash used, and methods of application must be studied.

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Some Effects of Cyclophosphamide (Cytoxan) on Normal Mice and Mice With L1210 Leukemia [1, 2]

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SUMMARY

The effects of *N,N*-bis-(β-chloroethyl)-*N′,O*-propylene phosphoric acid ester diamide, or cyclophosphamide, were evaluated in normal and leukemic mice. The overt manifestations of toxicity are described. Four days after an LD50 of cyclophosphamide, the bone marrow and spleen were aplastic. On the 6th day the marrow and spleen showed an unusual degree of megakaryocyte hyperplasia. Cyclophosphamide has been shown to be active against advanced L1210 leukemia. The effectiveness of the drug was markedly dependent upon the treatment regimen, but was independent of the route of administration. Treatment once weekly produced considerably greater increases in median survival than a daily or a 3 times weekly schedule. A regimen of treatment every 11th day was as effective in prolonging survival as a once weekly regimen, but a higher dose was required. Cyclophosphamide was much more effective than amethopterin or nitrogen mustard in prolonging the survival of mice with advanced L1210 leukemia when the optimum treatment regiment for each agent was used. The 6-mercaptopurine-resistant leukemia (L1210/AG-R) was as susceptible to the antileukemic action of cyclophosphamide as the parental L1210 strain.—J. Nat. Cancer Inst. 23: 1347–1359, 1959.

THE SEARCH for antitumor agents with enhanced chemotherapeutic activity has resulted in the synthesis of compounds with unusual chemical structures. One such material is *N,N*-bis-(β-chloroethyl)-*N′*, *O*-propylene phosphoric acid ester diamide, cyclophosphamide,[6] which was synthesized by Arnold and Bourseaux (*3*) and shown to possess activity against rat tumors (*4–6*).

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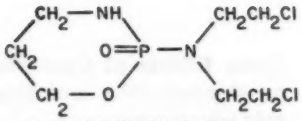
[2] Preliminary reports (*1, 2*) of portions of this work were presented at the April 1959 meetings of the American Association for Cancer Research and the Federation of American Societies for Experimental Biology, Atlantic City, N.J.

[3] The author is indebted to Mr. Marvin Schneiderman, for his advice and his help with the statistical evaluations, and to Mr. Sidney T. Yancey, for excellent technical assistance.

[4] Clinical Pharmacology and Experimental Therapeutics Service.

[5] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[6] Cyclophosphamide (generic name) has been variously known as B-518, Endoxan, and Cytoxan®. The cyclophosphamide used in these studies was generously supplied by Dr. Warren M. Cox, Jr., Technical Director of Laboratories, Mead Johnson & Company.



CYCLOPHOSPHAMIDE

The present report deals with the effects of cyclophosphamide in normal and leukemic mice. It demonstrates that treatment with this agent, at widely spaced intervals, is considerably more effective than other schedules in prolonging the survival of mice with advanced L1210 leukemia, and that cyclophosphamide is more active than amethopterin or nitrogen mustard in this system. The activity of cyclophosphamide against the azaguanine-resistant subline of L1210 leukemia (L1210/AG-R), which is cross resistant to 6-mercaptopurine, is also presented.

MATERIALS AND METHODS

Male DBA/2JN and CDBA (BALB/cAnN $\times$ DBA/2JN)F_1 mice from 2 to 3 months of age were used. The L1210 and L1210/AG-R leukemias [7] were carried at weekly intervals by intraperitoneal injections of 0.1 ml. of a 1:10 dilution of ascitic fluid in Locke's solution, into DBA/2JN and CDBA mice, respectively. This inoculum contained approximately 1.5×10^6 cells. Animals used for chemotherapy studies were given similar inoculations subcutaneously in the right axillary region. By this technique, DBA mice implanted subcutaneously with L1210 leukemia have median survivals of 9 to 11 days. CDBA mice bearing the L1210/AG-R leukemia have median survivals of 11 to 14 days. These tumors give 100 percent "takes" in recipients, and spontaneous regression has not been observed. Just prior to treatment animals were examined to assure the presence of a palpable tumor and were allocated to a particular treatment schedule and dose, by random assignment. The doses, routes, and schedules of drug administration are indicated in "Results." Mice were weighed prior to each treatment and routinely 3 times weekly.

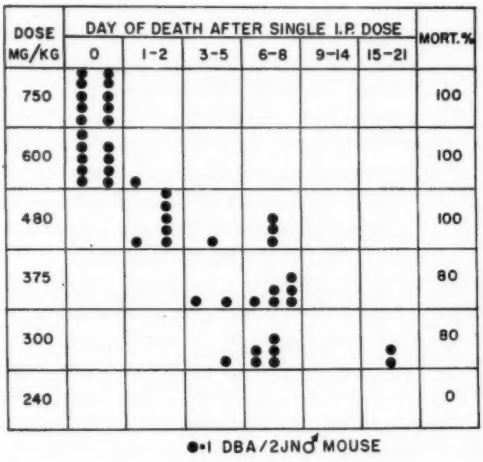
Cyclophosphamide and nitrogen mustard hydrochloride (Mustargen®, Merck Sharp & Dohme Company) were dissolved in 0.9 percent saline and amethopterin (American Cyanamid Company) in 2 percent aqueous sodium bicarbonate. Fine suspensions of 6-mercaptopurine (Burroughs Wellcome & Co.) were made in 1 percent aqueous carboxymethylcellulose. Nitrogen mustard solutions were injected within 10 minutes of preparation and other agents within 1 hour. Drug doses were given in a volume of 0.01 ml. per gm. of mouse body weight. In these studies LD50's were computed by Karber's method as modified by Cornfield and Mantel (*7*). Analysis of the results of treatment in the survival studies was accomplished by the nonparametric method of Festinger (*8*). A probability (P) of <0.05 was considered a significant difference between groups.

[7] The leukemias used in these studies were originally obtained from Dr. Lloyd Law.

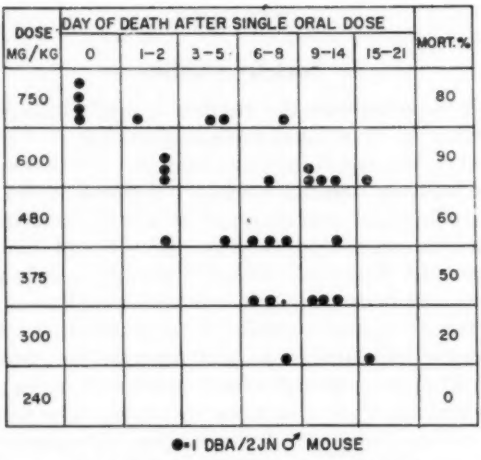
RESULTS

Toxicity Studies

Text-figure 1 demonstrates the results of single intraperitoneal doses of cyclophosphamide to normal mice. With 600 to 750 mg. per kg., deaths occurred on the day of injection (day 0). Within a few minutes of injection these animals became markedly depressed. In about 1 hour the mice had ruffled hair and demonstrated hyperactivity for 30 to 40 minutes, when depression again supervened. Respirations became progressively slow and labored. Hind-leg paralysis, followed by severe tonic and clonic convulsions, urination, defecation of loose stool, and respiratory arrest, preceded death within 5 to 10 minutes. At lower toxic doses, the toxicity was similar to that reported for nitrogen mustard. The drug was less toxic on oral administration (text-fig. 2), and death during the 2d and 3d week was more common. The manifestations of toxicity were similar to those observed after intraperitoneal injection. Four days after an intraperitoneal dose of 300 mg. per kg. there was severe depression of myeloid and erythroid elements in the bone marrow. Megakaryocytes, however, appeared to be preserved in number and morphology in the marrow (fig. 1) and in the spleen (fig. 2). On the 6th day after injection there was considerable repopulation of the marrow and spleen, and megakaryocytes were especially abundant (figs. 3 and 4). Many of these appeared to be young cells, and mitoses were fairly common. Detailed pathology will be the subject of a later report.



TEXT-FIGURE 1.—Toxicity of single intraperitoneal doses of cyclophosphamide. Ten mice were injected on day 0. LD50 = 300 mg. per kg., 95 percent confidence limits: 277 to 325 mg. per kg. Slope = 13.72.

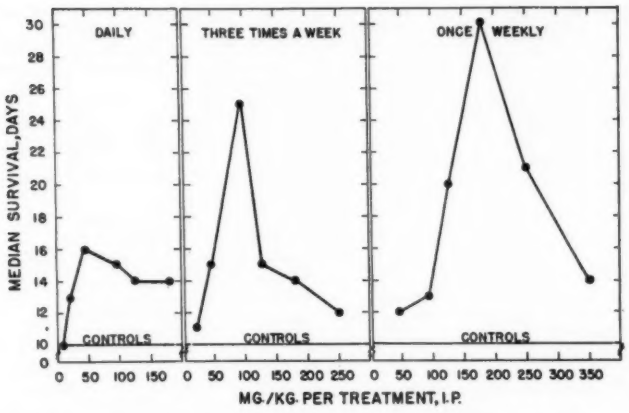


TEXT-FIGURE 2.—Toxicity of single oral doses of cyclophosphamide. Ten mice at each dose level were given a single oral administration on day 0. LD50 = 429 mg. per kg., 95 percent confidence limits: 375 to 491 mg. per kg. Slope = 6.26.

Tumor Studies

The effect of cyclophosphamide on the survival of leukemic animals is shown in text-figure 3. In this study treatment was begun 5 days after tumor implantation. The advantage of once weekly treatment is apparent. Statistical analysis has shown that the extension of survival beyond the controls, which results from each treatment schedule, is significant ($P<0.01$). Comparison of the results indicated that 3 times weekly and once weekly treatments were more effective than daily treatments ($P<0.01$), and that the once weekly schedule was more effective than the 3 times weekly schedule ($P<0.05$) and produced a 200 percent increase in median survival beyond that of controls. It is notable that several mice from the weekly treatment group were alive 5 weeks after tumor implantation. The last survivor, killed on the 49th day, had an enlarged spleen. A 1:10 by weight suspension of this spleen was made in Locke's solution, and 0.1 ml. injected intraperitoneally into each of 10 normal mice. None of the recipients developed leukemia.

A further exploration of treatment regimen is shown in table 1. Injections were begun 7 days after tumor implantation. Again once weekly treatment gave a significant increase in median survival compared to daily treatment ($P<0.01$). Discontinuation of treatment after either 4 daily doses or 2 weekly doses is also shown. This procedure did not modify the survival of these groups significantly ($P>0.05$) when compared to groups on similar regimens, which were treated until all mice succumbed. A schedule of injection every 11th day was more effective than daily treatment ($P<0.01$). Although the median survival of animals on this schedule (111% increase beyond controls) was less than those on the



TEXT-FIGURE 3.—The influence of treatment regimen on the survival of mice with established L1210 leukemia. Treatment was instituted 5 days after subcutaneous tumor implantation. Each point represents the median survival of a group of 10 mice; 20 mice were in the control group.

weekly schedule (145% increase beyond controls) the difference was not significant ($P>0.05$). Also, the apparent optimum dose was higher when the mice were treated every 11th day.

TABLE 1.—Effect of dose regimens of intraperitoneal injections of cyclophosphamide on survival of mice with 7-day subcutaneous implants of L1210 leukemia*

Drug	Apparent optimum dose (*mg./kg.*)	Dose regimen	Range in survival (*days*)	Median survival (*days*)	Increase in median survival beyond controls (*percent*)
Cyclophosphamide	90	Daily	8–18	16	78
"	90	Daily Days 7–10	10–18	16	78
"	180	Once weekly	16–28	22	145
"	180	Once weekly Days 7, 14	16–32	21	133
"	250	Every 11th day	18–31	19	111
Untreated	—	—	8–11	9	—

*Groups of 10 mice at 5 different dose levels were used for each schedule, with a control group of 20 mice.

An experiment to evaluate the influence of route of administration is shown in table 2. Once weekly oral, subcutaneous, and intraperitoneal treatments were equally effective and clearly more advantageous ($P<0.01$) than daily subcutaneous or intraperitoneal treatment. The optimum dose for oral treatment was higher than for either parenteral route when a weekly regimen was used.

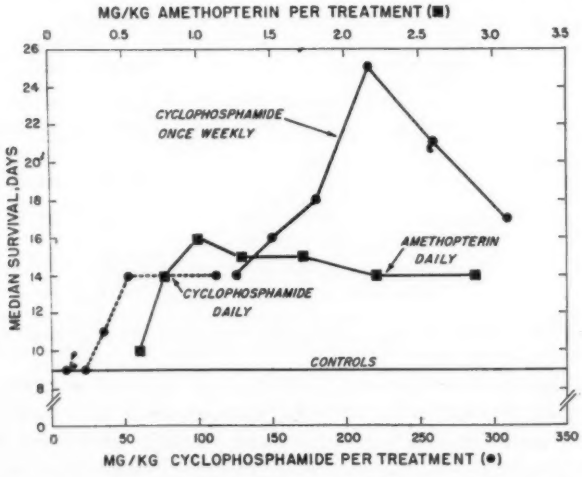
In view of the observed activity of cyclophosphamide, a direct comparison was made with the antifolic agent, amethopterin, in mice with

TABLE 2.—Influence of route and dose regimen of cyclophosphamide on survival of mice with 7-day subcutaneous implants of L1210 leukemia*

Drug	Apparent optimum dose (*mg./kg.*)	Dose regimen	Route	Range in survival (*days*)	Median survival (*days*)	Increase in median survival beyond controls (*percent*)
Cyclophosphamide	77	Daily	Subcutaneous	15–22	17	89
"	77	Daily	Intraperitoneal	9–19	18	100
"	215	Once weekly	Subcutaneous	15–29	26	189
"	215	Once weekly	Intraperitoneal	20–30	25	178
"	259	Once weekly	Oral	16–30	27	200
Untreated	—	—	—	9–11	9	—

*Groups of 8 mice at 5 different dose levels were used for each schedule, with a control group of 18 mice.

7-day-old implants of L1210 leukemia. This is illustrated in text-figure 4. The daily treatment regimen with amethopterin has been reported as optimal in advanced L1210 leukemia (*9*). Whereas daily treatment with cyclophosphamide was of the same order of effectiveness as amethopterin, once weekly treatment provided a substantially greater increase in median survival (178% beyond controls) than with the antifolic compound (78% beyond controls). This difference was significant at the 1 percent level. In another experiment (table 3) once weekly treatment with larger



TEXT-FIGURE 4.—Comparison of an optimum schedule of amethopterin with daily and once weekly schedules of cyclophosphamide, on the survival of mice with advanced L1210 leukemia. Treatment was instituted 7 days after subcutaneous tumor implantation. Each point represents the median survival of a group of 10 mice; 20 mice were in the control group.

doses of amethopterin was also compared with cyclophosphamide in mice bearing 7-day-old leukemia implants. A distinct advantage of daily over weekly amethopterin in advanced L1210 leukemia was observed ($P<0.01$). Again, once weekly treatment with cyclophosphamide was more efficacious than with daily amethopterin ($P<0.01$). The severity of the disease at the time treatment was begun is reflected in the early death of control mice.

TABLE 3.—Effects of cyclophosphamide and amethopterin on survival of mice with 7-day subcutaneous implants of L1210 leukemia*

Drug	Apparent optimum dose (*mg./kg.*)	Dose regimen	Route	Range in survival (*days*)	Median survival (*days*)	Increase in median survival beyond controls (*percent*)
Cyclophosphamide	180	Once weekly	Intraperitoneal	12–22	21	133
Amethopterin	40	Once weekly	Subcutaneous	11–12	11	22
Amethopterin	1. 7	Daily	Subcutaneous	9–19	15	67
Untreated	—	—	—	9–11	9	—

*Groups of 7 mice at 6 different dose levels were used for each schedule, with a control group of 15 mice.

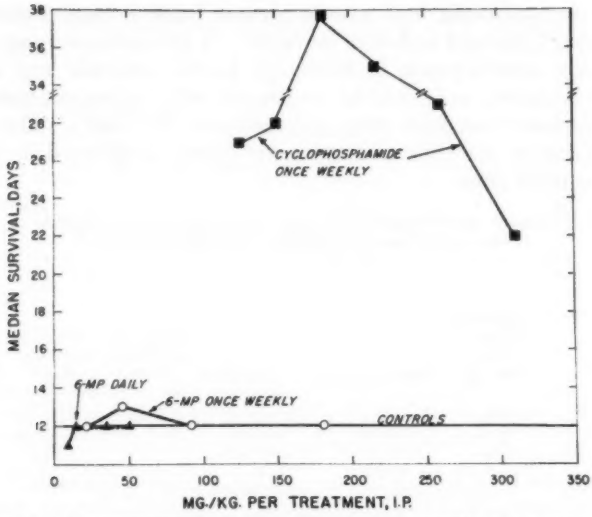
The effects of nitrogen mustard administered in several dose schedules were compared with those of cyclophosphamide injected once weekly. The results summarized in table 4 indicate the very limited increases in survival of leukemic mice treated intraperitoneally with nitrogen mustard in all the schedules employed—doses ranged from 0.31 to 10.0 mg. per kg. Treatment with cyclophosphamide resulted in a marked increase in median survival.

TABLE 4.—Effects of cyclophosphamide and nitrogen mustard on survival of mice with 7-day subcutaneous implants of L1210 leukemia*

Drug	Apparent optimum dose (*mg./kg.*)	Dose regimen	Range in survival (*days*)	Median survival (*days*)	Increase in median survival beyond controls (*percent*)
Nitrogen mustard	10. 0	Once weekly	7–11	10	11
"	10. 0	Every 4th day	8–12	11	22
"	2. 5	3 Times weekly	9–13	10	11
"	5. 0	Daily	8–12	11	22
Cyclophosphamide	180	Once weekly	16–28	22	145
Untreated	—	—	7–11	9	—

*Groups of 10 mice at 4 different dose levels were used for each schedule of treatment with nitrogen mustard and at 3 dose levels for treatment with cyclophosphamide; intraperitoneal injections. Control group of 19 mice.

A study with the 6-mercaptopurine-resistant leukemia (L1210/AG-R) is illustrated in text-figure 5. The resistance to daily treatment and to large single doses of 6-mercaptopurine is clear. Nonetheless, this tumor was quite sensitive to once weekly treatment with cyclophosphamide.



TEXT-FIGURE 5.—Comparison of the effects of cyclophosphamide and 6-mercaptopurine on the survival of mice with 6-mercaptopurine-resistant leukemia (L1210/AG-R). Treatment was begun 7 days after subcutaneous tumor implantation. Each point represents the median survival of 10 mice; 20 mice were in the control group.

DISCUSSION

Cyclophosphamide was synthesized to provide an alkylating agent in an inactive "transport form" which would be converted *in vivo* by enzymic or other processes to an "active form." It was hoped that in such an agent the oncolytic properties might be greater than those of available alkylating agents and that it might possess a large "therapeutic index" (*3*). Evidence has not yet been brought forward to indicate the mechanism of action *in vivo*. Until the detailed pharmacological disposition of cyclophosphamide is elucidated, suggestions regarding mechanism are purely speculative.

The lesser toxicity of this compound on oral administration to mice, reported here, was not found in studies of rats (*1*) in which oral and intraperitoneal toxicity were comparable. The overt acute manifestations of cyclophosphamide toxicity are in general similar to those described for nitrogen mustard (*10–15*). An interesting phenomenon was the interposition of a hyperactive stage of 30 to 40 minutes' duration between an initial prolonged and a final short period of depression. The terminal convulsions appeared related in time to respiratory paralysis and probably resulted from anoxemia. Delayed toxicity was quite similar to that reported for other alkylating agents (*12–17*), with the qualification that on oral administration delayed deaths were fairly common. Several clinical reports (*18, 19*) have stated that even when severe leukopenia

is produced by this drug, thrombocytopenia occurs infrequently and is only of moderate severity. The reason for this is uncertain. The marrow on day 4 resulting from an LD50 (300 mg./kg.) of cyclophosphamide (fig. 1) appeared to be more severely hypoplastic than the marrow seen in nitrogen mustard toxicity (*10*, *15*, *16*, *20*, *21*). The megakaryocytes seemed morphologically normal here and in the spleen (fig. 2). It is possible that the megakaryocytes which have remained are capable of platelet formation and may not be severely damaged. The marrow regenerated rapidly (fig. 3) and megakaryocytes in this tissue and in the spleen (fig. 4) were unusually abundant. Megakaryocytes in mitosis and young forms were numerous. This remarkable megakaryocyte hyperplasia might result in increased platelet production.

In the present investigations it has been demonstrated that spreading the interval of intraperitoneal drug administration from daily to 3 times weekly results in a considerable increase in median survival beyond that of controls. When this interval was increased to once weekly, a further increase in median survival was observed (text-fig. 3). In view of the possibility that a greater interval might have been still more effective, the dosage regimen was altered to every 11th day (table 1). No additional benefit attended this modification. It is apparent from the results of this experiment that a single dose of cyclophosphamide is very effective in prolonging the survival of mice with advanced L1210 leukemia. To see if excessive toxicity limited the efficacy of the weekly or daily dose schedule, treatment was discontinued after 2 weekly or 4 daily doses. Maximum survival with these regimens did not differ appreciably from results with continuous weekly or daily therapy (table 1). With this information, a schedule of weekly drug administration appears to be reasonably optimum as well as convenient. This has also been shown for some other alkylating agents (*22*, *23*).

The increases in survival obtained with daily subcutaneous and intraperitoneal injections of cyclophosphamide were comparable, but significantly less than those resulting from once weekly injections by these routes, in the same experiment (table 2). The drug was equally efficacious when administered once weekly by oral, subcutaneous, or intraperitoneal route. The doses producing maximum increases in survival were identical for subcutaneous and intraperitoneal routes (77 mg./kg. for the daily schedule and 215 mg./kg. for the once weekly regimen). A higher dose was required for the oral (259 mg./kg.) than for the parenteral (215 mg./kg.) once weekly schedule. This is consistent with the lower toxicity of the drug on oral compared to intraperitoneal administration in normal mice (text-figs. 1 and 2).

In a system using L1210 leukemia it has been shown that amethopterin is most effective on a divided schedule (every 4 days) when given shortly after tumor implantation, but that a daily schedule is more effective against advanced disease (*9*). The present studies, which used advanced L1210 leukemia, have confirmed the advantage of daily over weekly administration of this agent (table 3); the situation with cyclophospha-

mide was quite different. When cyclophosphamide and amethopterin were both given on a daily schedule, a greater increase in median survival was obtained with amethopterin (text-fig. 4). However, the administration of cyclophosphamide at its optimum schedule (once weekly) unequivocally demonstrated that this agent was much more effective than amethopterin in prolonging the survival of mice with advanced leukemia (text-fig. 4 and table 3). Cyclophosphamide was also far more effective against advanced L1210 leukemia than nitrogen mustard.

An antimetabolite-resistant tumor would not be expected, *a priori*, to show cross resistance to an alkylating agent. It appeared important to test this experimentally in view of the therapeutic problems posed by antimetabolite-resistant leukemia in clinical practice. The 6-mercaptopurine-resistant leukemia (L1210/AG-R) responded to treatment with cyclophosphamide (text-fig. 5) as well as the nonresistant leukemia had responded.

The present report emphasizes the desirability of administering chemotherapeutic agents of interest in several dosage schedules. Information obtained from such studies can indicate the activity of an agent against animal tumors more reliably than techniques that use a single treatment regimen and route. When striking differences are observed for various schedules of treatment, as with cyclophosphamide, the investigation of several therapeutic regimens at the clinical level would certainly seem advisable. The transition from a particular interval of treatment that is effective in animal systems to an appropriate treatment schedule for patients is difficult. Information derived from animals can at best suggest a pattern rather than a rigid formula. Whether the pattern of therapy with cyclophosphamide, shown to be most efficacious in mice (widely separated large doses), will be similarly beneficial in patients is yet to be determined.

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PLATES

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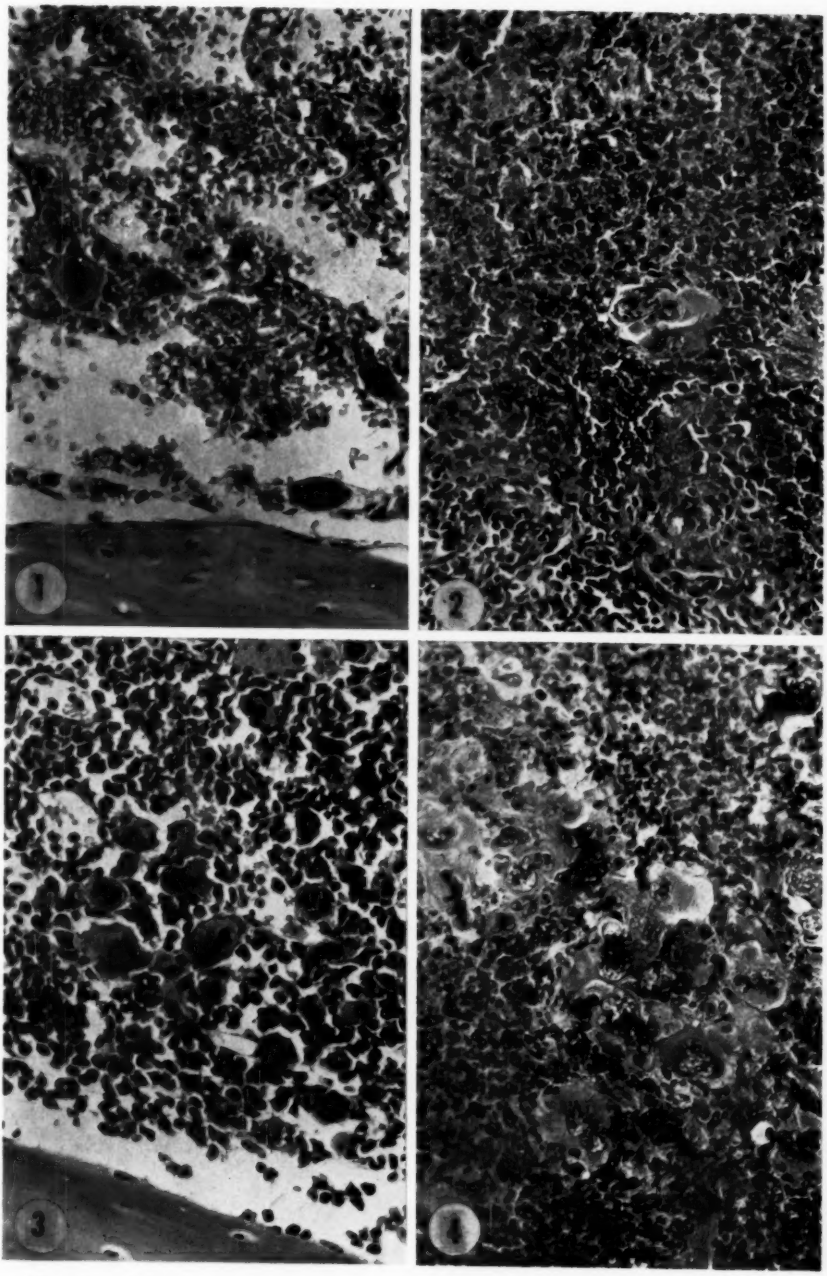
FIGURE 1.—Bone marrow from DBA/2JN mouse 4 days after an LD50 (300 mg./kg.) of cyclophosphamide. *Note* intact megakaryocytes in aplastic marrow. The majority of formed elements are red blood cells. Zenker-formalin fixative. Decalcified in formic acid. Hematoxylin and eosin. × 285

FIGURE 2.—Spleen from DBA/2JN mouse 4 days after an LD50 (300 mg./kg.) of cyclophosphamide. *Note* intact megakaryocytes. Zenker-formalin fixative. Hematoxylin and eosin. × 285

FIGURE 3.—Bone marrow from DBA/2JN mouse 6 days after an LD50 (300 mg./kg.) of cyclophosphamide. *Note* large number of megakaryocytes in regenerating marrow. Zenker-formalin fixative. Decalcified in formic acid. Hematoxylin and eosin. × 285

FIGURE 4.—Spleen from DBA/2JN mouse 6 days after an LD50 (300 mg./kg.) of cyclophosphamide. *Note* nests of megakaryocytes. Mitotic megakaryocytes seen in several other areas. Zenker-formalin fixative. × 285



LANE

526279—59——15

Quantitative Studies on Rous Sarcoma Virus. IV. An Investigation of the Nature of "Noninfective" Tumors Induced by Low Doses of Virus [1, 2, 3]

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SUMMARY

Tumors with no detectable virus content, termed "noninfective," have been found to comprise 10 to 20 percent of those tumors arising below the 50 percent dilution endpoint in 10-day-old chicks inoculated subcutaneously. These tumors did not have a significantly different growth rate when compared to other tumors initiated with equivalent doses of virus. These observations confirm those of Bryan, Calnan, and Mahoney. The cells of noninfective tumors did not produce detectable virus on the chorio-allantoic membrane of the chick embryo or after serial cultivation in tissue culture. Cultures derived from these tumors were resistant to challenge infection with Rous sarcoma virus. Serial transplantation of noninfective tumors resulted in the return of virus-containing tumors. In two instances this did not occur until after four transplant generations. Five noninfective tumors were biopsied and followed; all eventually regressed. Five of 27 virus-producing tumors similarly followed also regressed. There was no correlation between neutralizing antibody levels and virus content, or regression, of tumors. Birds with multiple tumors initiated with less than one tumor-inducing dose of virus were observed carrying both noninfective and virus-producing tumors. These observations were interpreted as suggesting the existence of a form of cell-virus integration in which infectious virus is only rarely produced.—J. Nat. Cancer Inst. 23: 1361–1381, 1959.

"MASKING" IN relation to tumor viruses may be thought of as an unexplained reduction in virus content of tumors. In its extreme, masking of a tumor virus is associated with an inability to demonstrate infectious virus at all. This extreme has been frequently observed in the Rous sarcoma. Early passages of chicken tumors frequently went through unexplained "nonfilterable phases" (*1*, *2*). Carr observed a definite correlation between age of tumors at time of harvest and virus content. Tumors harvested more than 40 days after inoculation were invariably free of detectable virus (*3*). Duran-Reynals and Freire found a similar

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[2] These studies were supported by research grants from the American Cancer Society, Inc., and from the Jane Coffin Childs Memorial Fund for Medical Research.

[3] A preliminary report of these studies was presented at the Seventh International Cancer Congress, London, 1958.

[4] The able technical assistance of Mrs. Mary McGill and Mrs. Loraine O'Neill is gratefully acknowledged.

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correlation between decreased virus yield and age of the tumor-bearing host (*4*).

A most striking example of this phenomenon was reported by Bryan, Calnan, and Maloney (*5*). These investigators observed, within a certain range, a marked correlation between inoculum dose and virus content. Four of seventeen tumors induced by less than one ED50 dose contained no demonstrable virus. These findings have been confirmed by Groupé and Rauscher (*6*) in similar studies carried out by the intracerebral route in 1-day-old chicks.

The mechanism of these forms of virus masking has so far not been determined. Numerous studies have demonstrated the presence of virus-neutralizing inhibitors in avian neoplasms, at least some of which are probably identical with virus-neutralizing antibody. The possible role of such inhibitors in the masking process has been reviewed recently (*7*). That such inhibitors account for at least some examples of virus masking would appear to be beyond question. Heat-stable inhibitors have been demonstrated in tumors induced by small doses of virus in turkeys (*8*). It has, however, been reported that such inhibitors are not in all tumors which are devoid of demonstrable virus (*9*). Their role as the fundamental cause of the virus masking in this instance is thus by no means established.

Since naturally occurring forms of virus-induced neoplasia probably originate in "low doses" of virus, it is likely that attempts at isolation of etiologic agents from such tumors will have to consider the possibility of virus masking. It is therefore pertinent to examine known experimental examples of this phenomenon in considerable detail.

The "low-dose" tumors produced by Bryan appeared to offer a reproducible system for such a study. This report will summarize studies on the pathogenesis and nature of tumors, induced in the wing web of 1- to 2-week-old birds by minute doses of virus, which do not contain extractable infective virus. These tumors will be termed "noninfective." This term is recognized to be somewhat arbitrary since it appears likely that rare cells within these tumors may occasionally produce virus.

It will be demonstrated that virus-neutralizing antibody does not contribute to this masking. Evidence will be described which supports the hypothesis that the lack of virus production is a consequence of a nonproductive form of cell-virus integration.

MATERIALS AND METHODS

Virus stocks.—Stable stocks of virus derived from the line of chicken tumor #1, which had been carried in the laboratory of Dr. F. Duran-Reynals, were used in these experiments. Tumors were produced by inoculation of concentrated extracts into the wing web of 10- to 14-day-old chickens and were harvested 6 to 9 days after inoculation. Tumors that had been frozen at −70° C. and stored at −40° C. for short periods were

homogenized without thawing at a 10 percent concentration in tissue-culture medium containing 0.001 percent testicular hyaluronidase (Wyeth). Homogenization was carried out for 2 minutes in a high-speed Servall homogenizer held in an ice-water bath. The resulting extracts were held for 4 hours at 4° C., and then clarified by centrifugation for 15 minutes at 2,000 × g. Supernatants were frozen in sealed ampoules at −70° C. and stored in a dry-ice chest.

Chickens.—All animals in these experiments were of the "Cornucopia" White Leghorn strain, previously identified as the "Pure Hope line" (*10*), supplied by Mt. Hope Poultry Farm, Williamstown, Massachusetts. Chickens were inoculated at 1 to 2 weeks of age.

Determination of virus content of tumors.—Since it was of interest to examine tumors for virus content as early as possible after virus inoculation to minimize the role of neutralizing antibody, it was decided to harvest tumors as soon as possible after they reached a diameter of 5 mm. Palpation was generally carried out at biweekly intervals, thus most tumors ranged between 5 and 10 mm. at the time of harvest.

Tumors were homogenized at a 10 percent concentration (wt./vol.) in 0.05 M citrate buffer, *p*H 7.0, containing 0.001 percent testicular hyaluronidase, 2 percent inactivated horse serum, and 50 units of penicillin and 50 μg. of streptomycin per ml. Homogenization was carried out in motor-driven, teflon Ten Broeck-type homogenizers at 0 to 2° C. for 3 minutes. Extracts were clarified by centrifugation at 2,000 × g for 10 minutes, frozen in alcohol and dry ice, and stored at −40° C. for short periods prior to assay. Dilutions for virus assays were made in the diluent described. Virus assays were carried out by the chorio-allantoic membrane (CAM) pock-count method, which has been described in detail (*10*).

Determination of neutralizing antibody content of serums.—All serums were inactivated at 56° C. for 30 minutes prior to testing. Serums were incubated for 30 minutes at 37° C. with equal volumes of virus stock diluted to contain 200 pock-forming units (p.f.u.) per 0.1 ml. Normal, inactivated horse serum was used in control mixtures. After incubation, the mixtures were held at 4° C. prior to inoculation. Neutralization indexes were calculated by subtraction of the log p.f.u. in the test mixture from the log p.f.u. in the control mixture.

Tissue-culture techniques.—Tumors to be grown in tissue culture were minced into small fragments, which were washed once with phosphate-buffered saline (PBS) lacking calcium and magnesium (*11*), prior to suspension in PBS containing 0.2 percent trypsin (Difco 1:300) and 0.001 percent testicular hyaluronidase. Several successive cycles of trypsinization were carried out at 37° C. on a magnetic stirrer for 10 to 20 minutes. The supernatant cell suspensions were immediately diluted with an equal volume of a tissue-culture medium composed of Eagle's basic medium (*12*) containing 5 percent duck serum and 5 percent calf serum. All serums were inactivated prior to use and were free of virus-neutralizing activity.

Trypsin was removed by slow-speed centrifugation and the cells resuspended in complete medium. Cells were seeded into 2-ounce prescription

bottles at a concentration of 10^6 cells, per bottle, in 5 ml. of medium. Cultures were routinely fed at 2- to 3-day intervals and were subcultured whenever cell sheets became confluent. Subcultures were carried out by the removal of cells from the glass with 0.1 percent trypsin in PBS, without divalent cations, at 37° C. after a preliminary washing of the cell sheet with PBS. Cells were immediately diluted with medium after trypsinization and resuspended in fresh medium prior to reseeding fresh bottles with 2×10^5 cells. Citric acid nuclei counts (*13*) were used for measurements of cell multiplication.

The measurement of "infectious centers."—To determine the proportion of cells which can release virus on the CAM (infectious centers), cultures were washed twice, and then overlayed for 2 hours with Eagle's medium containing 10 percent inactivated pooled chicken serum, before trypsinization. This medium neutralized more than 99 percent of 200 p.f.u. per ml. when mixed in equal volume and incubated for 30 minutes at 37° C. After trypsinization, cells were washed twice in standard medium to remove residual inhibitor. Dilutions containing known numbers of cells were then inoculated onto the CAM by the standard procedure. Aliquots of each dilution were also inoculated after three cycles of quick freezing and quick thawing at −70° C. and 37° C., since it was found that this did not inactivate free or cell-associated infectious virus, but did destroy the ability of cells to liberate virus.

Infectious center measurements were considered valid only if freezing and thawing destroyed more than 75 percent of the pock-forming ability of the cell suspension tested.

Challenge infection of cells cultivated in vitro.—Cultures in the exponential growth phase, containing approximately 5×10^5 to 1×10^6 cells, were infected by adsorbing 0.2 ml. of high-titer virus stocks ($10^{6.5}$–10^7 p.f.u./ml.) onto the monolayers for 2 to 3 hours at 37° C. After virus adsorption, excess virus was removed by three successive washes with standard medium, and the cultures were refed. At intervals thereafter, 1-ml. aliquots of culture medium were removed, replaced by fresh medium, frozen at −70° C. and held at −40° C. until assayed. Preliminary virus-growth experiments had revealed there to be at all times an average of 10 to 20 times as much virus in the medium as was associated with the cells, so that it was not necessary to sacrifice cultures for these studies.

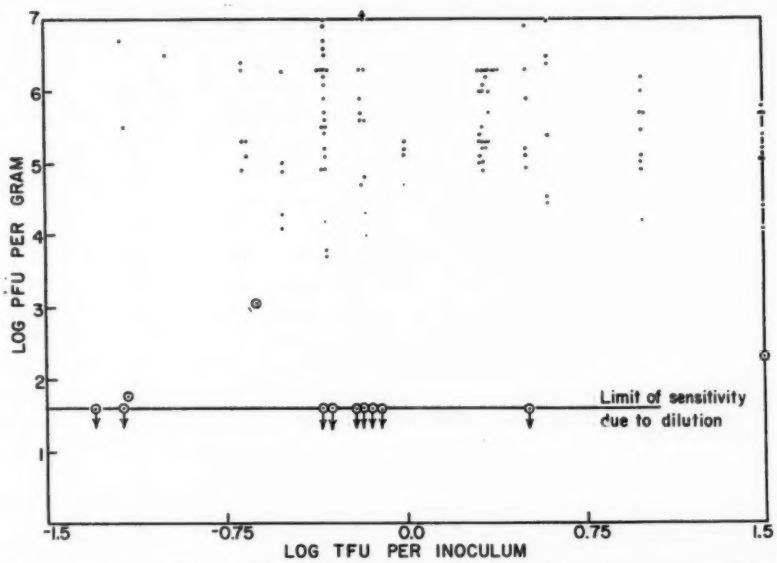
For control experiments chick-embryo fibroblasts were prepared by the method of Dulbecco (*11*) from 10-day-old embryos. These were infected during their second passage *in vitro*.

Transplantation of "noninfective" tumors.—Tumors were harvested with sterile precaution into petri dishes containing tissue-culture medium. After mincing with scissors, 1- to 2-mm. fragments were transplanted, with the aid of 18-gauge trocars, into the subcutaneous space of the wing web of 1- to 2-week-old birds. Separate, autoclaved instruments were used for each tumor. In an effort to rule out possible cross contamination between virus-producing and noninfective tumors, each group of recipient birds was housed in a separate cage.

EXPERIMENTS

I. Effect of Virus Dose in the Low-Dose Range on Virus Content of Tumors

Since the original report by Bryan and his coworkers (*5*) had been based on only 17 tumors in the range of inoculum potency that resulted in less than 50 percent tumor incidence, it appeared desirable to accumulate additional data relating dose to virus content of tumors in this range. Text-figure 1 summarizes the virus content of 122 tumors initiated by small doses of virus in the wing web of chickens 1 to 2 weeks of age at the time of inoculation.



TEXT-FIGURE 1.—The relation between dose of Rous sarcoma virus inoculated and virus contents of tumors. Each point represents a single tumor. One t.f.u. (log t.f.u.=0.0) is the dose producing tumors in 50 percent of inoculated animals as estimated by the method of Reed and Muench (*14*). Since 0.1 cc. of a 10 percent extract of tumor was inoculated onto 5 membranes for assay of the undiluted samples, and since on the average only half the inoculum reaches the CAM due to the method of inoculation (*10*), less than 40, or 1.6 log, p.f.u. per gm. of tumor could not be detected.

It is of interest to note that 8 of the 9 noninfective tumors occurred below the 50 percent dilution endpoint. The majority of the remaining tumors contained large but very variable amounts of virus, though 3 had a small but still measurable virus content. A small proportion of tumors occurring below the 50 percent endpoint contained near-maximal amounts of virus.

Table 1 summarizes the harvest data from this series of noninfective tumors and from a comparable series of virus-containing tumors arising

below the 50 percent endpoint. Due to the use of biweekly palpation intervals, the time elapsing between palpation and tumor harvest is only a rough estimate of the time elapsing between the appearance of the tumor and its harvest. Table 1 reveals no difference between the average harvest times, estimated tumor latent periods (time between virus inoculation and appearance of tumor), or tumor size at time of harvest between the virus-containing and the noninfective group. These data are quite comparable with those previously observed by Bryan *et al.* (*5*) whose analysis also failed to reveal a relation between these variables and virus content.

TABLE 1.—Harvest data for tumors arising below 50 percent dilution end point

Tumor No.	Virus content (*log p.f.u./gm.*)	Tumor harvest		
		After virus inoculation (*days*)	After palpation (*days*)	Mean diameter (*mm.*)
1	<1.6	19	3	5
2	"	27	9	10
3	"	27	14	8
4	"	26	13	9
5	"	18	5	8
6	"	15	3	6
7	"	15	4	7
8	"	23	6	5
9	"	23	8	8
Average	<1.6	21.4	7.2	7.3
10	6.3	18	7	9
11	4.3	18	5	8
12	4.0	27	14	9
13	7.3	22	4	7
14	5.3	18	4	6
15	5.1	28	14	7
16	1.8	28	14	8
17	5.5	18	4	8
18	6.3	18	4	10
19	5.6	22	4	7
20	4.7	18	5	6
21	5.9	26	8	5
22	6.3	18	4	11
23	5.3	26	10	6
24	6.3	18	4	10
25	6.7	26	12	7
Average	5.4	21.8	7.3	7.7

The findings suggested the hypothesis that the virus content of these tumors was determined by the nature of the initial virus-cell interaction rather than by a form of host response. It was postulated that a small proportion of single cell-virus encounters might result in noninfective tumors. If the tumors depended on an intrinsic property of this encounter, it would then be expected that the incidence of such tumors would decline very rapidly as the dose increased to the level where tumors originated in multiple foci.

That tumors arising below the 50 percent dilution endpoint originate in single foci is suggested by their morphological appearance. When observed early in their development, they are almost invariably single

and spherical. Furthermore, at slightly higher dose levels, multiple focal tumors or irregularly shaped single tumors are observed.

II. The Nature of Noninfective Tumors

A. Absence of virus production in vitro.—To determine whether "unmasking" of virus might occur when tumors were removed from the host environment, 6 of the noninfective tumors, as well as a larger number with intermediate and high virus contents, were trypsinized and placed in tissue culture. It was anticipated that if a product of host immune response were responsible for the low, or absent, virus content of noninfective tumors, this should be rapidly diluted out during growth *in vitro*, so that virus production might be observed.

Only 4 of the 6 noninfective tumors survived the first tissue-culture transfer. The growth of the most vigorous is shown in table 2. It will be seen that no virus appeared in the supernatant of frozen and thawed cultures of this tumor. Similar results, though under less optimal conditions of cell growth, were observed with the remaining noninfective tumors. Table 2 also summarizes for comparison the results obtained with two virus-producing tumors. The first, which like most Rous sarcomas cultured under the conditions used, grew well for only two passages, was of intermediate virus content. This is reflected in the intermediate level of virus production *in vitro*. The third tumor, whose cultivation is described in table 2, represented an unusual example in that it was the only tumor which survived serial subcultivation for an extended period. This line required weekly transfers for 10 successive weeks prior to undergoing giant-cell formation and death. A high level of virus production was observed throughout this period.

TABLE 2.—Spontaneous virus production by cultures prepared from Rous sarcomas

Virus content of tumor (*log p.f.u./gm.*)	Tissue-culture passage*	Days after transfer	Nuclei per flask†	Virus content of culture (*log p.f.u./ml.*)‡	Proportion cells "Infectious centers"
<1.6	1	4	8×10^5	<1.0	$<10^{-4.4}$
	2	3	2×10^5	<1.0	
		6	1.2×10^6	<1.0	$<10^{-5.4}$
	3	3	4×10^5	<1.0	
		7	1.5×10^6	<1.0	
	4	3	3×10^5	<1.0	
		6	5×10^5	<1.0	
5.26	1	4	2×10^6	3.59	$10^{-2.3}$
	2	7	8×10^5	3.31	
7.30	3	6	1.6×10^6	5.80	$10^{-0.26}$
	9	6	4×10^5	5.26	

*Subcultures initiated with 1 to 2×10^5 cells per culture in 5 ml. of medium.
†Citric acid nuclei counts.
‡Cultures frozen and thawed 3 times (−70° C. and 37° C.) prior to assay.

Experiments were carried out to determine the proportion of cells in cultures that can give rise to pocks on the chorio-allantoic membrane before, but not after, freezing and thawing. Such cells, which may be termed infectious centers, owe their pock formation to their ability to produce virus on the CAM (*15*). The results of several such determinations are presented in the last column of table 2. Pocks were never observed when cell suspension of noninfective cultures were used. With virus-producing tumors, there appeared to be a close correlation between the level of virus in tissue cultures and the number of virus-producing cells, as measured by this method. In general it has been found that each infectious center is associated with between 1 and 5 p.f.u. in the supernatant (or whole culture, frozen and thawed).

It thus appears that less than 1 in 10^5 to 10^6 cells derived from noninfective tumors releases virus under these conditions. Unmasking was not observed.

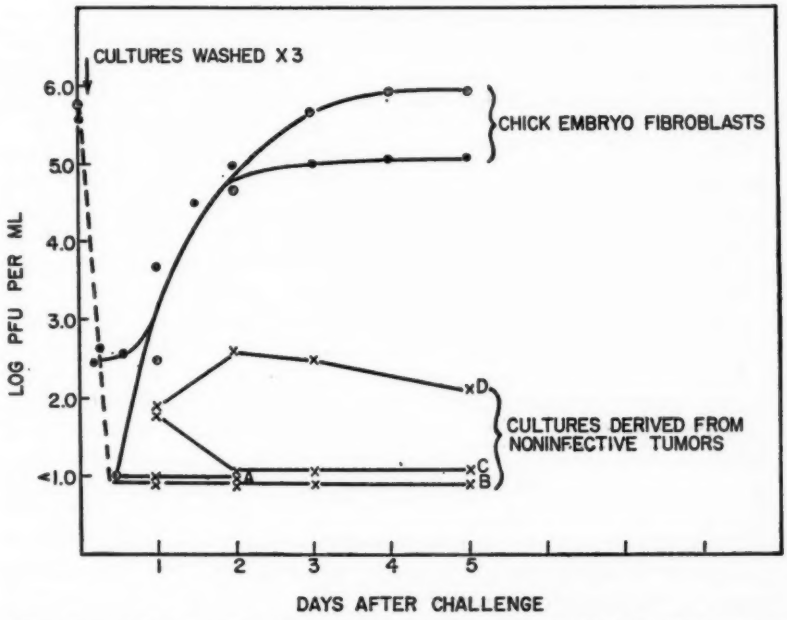
Unfortunately it is impossible to evaluate conclusively the extent of possible selection for normal cells which could occur in such experiments. The rapid growth of the initially planted cells in the cell lines described in table 2 is somewhat against such selection.

B. Susceptibility of cells, derived from noninfective tumors, to challenge infection with RSV in vitro.—To gain further insight into the nature of the cells contained in cultures derived from noninfective tumors, a series of experiments were carried out to determine their susceptibility to challenge infection. These are summarized in text-figure 2.

The top two curves represent infections of normal chick-embryo fibroblasts during their second passage *in vitro*. The upper curve of the two represents the highest degree of susceptibility observed in a large number of similar experiments. Such a curve is associated with transformation of 50 to 100 percent of cells into infectious centers. The second curve is a more standard result, associated with the transformation of approximately 10 percent of cells into infectious centers.

Curves A, B, and C represent infections of second tissue-culture passages of 3 noninfective tumors. The lack of measurable virus production suggests that less than 1 in 100,000 cells has responded to challenge infection in these cultures. Curve D represents an infection of a third tissue-culture passage derived from a noninfective tumor. In this case it would appear that approximately 1 in 5,000 cells responded by producing virus.

If chick-embryo fibroblasts are indeed an adequate control, these experiments would suggest that cultures derived from noninfective tumors are composed predominantly of cells that resist infection by RSV. Such resistance might imply that these cells are already infected, in a stable manner, with the Rous genome. It must be recognized, however, that such an interpretation is highly speculative in the absence of studies on the appropriate control, which ideally would be derived from normal stromal fibroblasts surrounding the noninfective tumor of the 2- to 3-week-old chicken.



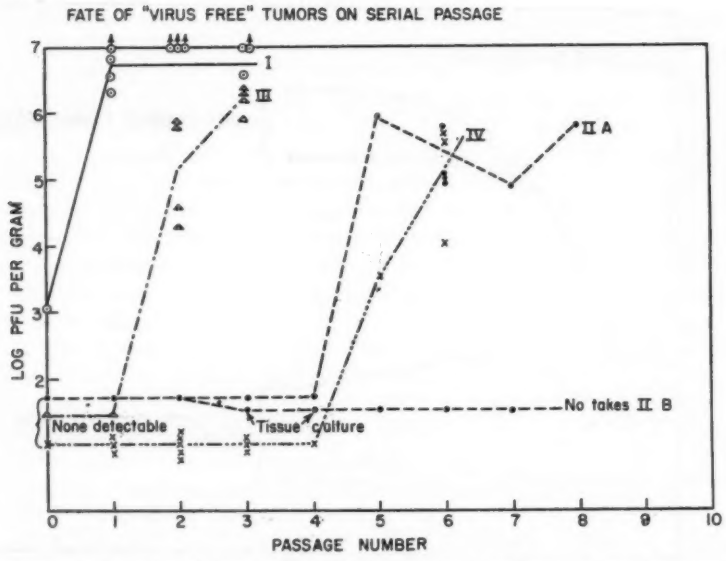
TEXT-FIGURE 2.—Growth of Rous sarcoma virus on normal chick fibroblasts and on cells derived from "noninfective" tumors. Curves A, B, and C represent infection of second tissue-culture passages of such tumors. Curve D derives from infection of the third tissue-culture passage of cells used in C.

C. Passage of noninfective tumors in vivo.—In the hope of obtaining a stable supply of noninfective tumors, attempts were made to maintain such tumors by serial transplantation *in vivo*.

A similar attempt has been reported by Calnan *et al.* (*16*). These investigators originated their transplantation line with a tumor which contained a measurable, though small, quantity of virus. At each passage, slow-growing tumors were selected. Transfers were made at 2- to 4-week intervals. Although occasional tumors were free of demonstrable virus, persistent absence of virus could not be achieved by these methods. Because of these results, somewhat modified procedures were used in the experiments summarized in text-figure 3.

Since tumors with measurable virus have always been observed to rise to levels of high virus content after transplantation (line I, text-fig. 3), an attempt was made to originate lines of tumors containing no detectable virus. Furthermore, due to the observation that noninfective tumors regress in the primary host, and after transplantation, short intervals of 5 to 7 days between transfers were used. Despite these modifications, it was not possible to maintain the noninfective tumors without the appearance of tumors containing detectable virus.

Two of the five noninfective tumors which were transplanted failed to



TEXT-FIGURE 3.—Fate of three noninfective tumors and one tumor of low, but measurable, virus content, on serial passage *in vivo*.

take in any of the birds inoculated. The fate of the remainder, which were transplantable, is summarized in text-figure 3.

Line II originated in a 5-mm. firm tumor, which was removed 19 days after virus inoculation and 3 days after appearance of the tumor. The first passage resulted in 3 vigorous tumors in each of 3 birds inoculated, averaging 9 mm. in diameter 6 days after transplantation. Two of these were assayed and were found to be free of detectable infectivity. One tumor served as the source for the second passage. Again 3 tumors appeared in the 3 birds inoculated. These averaged 10 mm. in diameter on the 7th day. Two were utilized to set up tissue cultures; the third was used for assay (no virus detectable) and transplantation. Of 3 birds inoculated in the third passage only 1 developed a growing nodule. This reached a size of 2 × 5 mm. and was harvested for assay (no virus detectable) and transplantation. Again in the following passage only 1 bird developed a growing tumor. This was harvested 8 days after transplantation when it had reached an average diameter of 4 mm. Six birds were inoculated with 1-mm. fragments of this material. Only 1 developed a tumor, which appeared 11 days after transplantation and grew progressively into a hemorrhagic virus-containing tumor.

Material from the second passage, which had been passed twice in tissue culture without production of detectable virus, was reinoculated into 1 bird, Line II B. The inoculum consisted of a "rolled up" monolayer containing approximately 2 to 4 × 10^6 cells. A nodule appeared which reached an average diameter of about 4 mm. after 5 days. This and sub-

sequent transplants were seen on histological examination to be composed largely of lymphoid reactive tissue. Since clear-cut tumor tissue could not be identified with certainty, it is possible to conclude from this series only that virus was not produced by the originally transplanted cells.

Line III originated in a tumor which was biopsied for assay and transplantation 27 days after virus inoculation, and 9 days after its appearance. At the time of biopsy the tumor had reached a mean diameter of 10 mm. Six days after transfer each transplanted bird had a tumor about 8 mm. in diameter. These were composed of soft, light-tan tissue which histologically was composed of loosely packed, elongated fibroblastic cells in a somewhat myxoid stroma. It is noteworthy that margination of chromatin and prominence of nucleoli, which are two hallmarks of standard Rous sarcoma cells (*17*), were extremely rare in passage lines of the noninfective tumors that have been examined. In fact, these cells could be classified as neoplastic only by their growth behavior and histological arrangement, and not by their cytologic characteristics.

Three noninfective tumors were examined histologically prior to transplantation; two were indistinguishable from typical Rous sarcomas. These tumors showed considerable cellular anaplasia. Lymphoid nodules were present in the periphery of these tumors to no greater extent than has been seen in the majority of virus-producing tumors. One noninfective tumor was composed solely of vacuolated giant tumor cells, most of which appeared to be in the process of degeneration.

Line IV reiterated the observations made on the previous lines. Only in the first transplant generation could definite Rous tumor be histologically identified. Subsequent tumors appeared to be composed largely of reactive tissue. The only evidence for continued transplantation of tumor cells was the appearance of virus-containing tumors in the fifth passage.

III. Genesis of Noninfective Tumors

A. Role of virus-neutralizing antibody.—The lack of unmasking of noninfective tumors after cultivation *in vitro*, and in three instances after one or more passages in young chickens highly susceptible to RSV, suggested that the absence of detectable virus in these tumors was an intrinsic cellular property and did not depend on an unusual degree of immune response by certain birds. To investigate further this latter hypothesis two experiments were carried out in which determinations of serum antibody could be correlated with the virus content of tumors. In both experiments, tumors were biopsied so that information regarding their fate in the primary host might be obtained and similarly correlated with their virus content and with the neutralizing antibody response of their hosts.

In the first experiment, the results of which are summarized in table 3, a series of tumors occurring below the 50 percent dilution endpoint in a bioassay experiment were selected for biopsy and further study. The nine selected tumors were not a random sample in that they were selected on the basis of lack of hemorrhage and firmness to palpation in the hope

that a high proportion of noninfective tumors would be included. It should be noted that such selection raised the yield of such tumors, but that it was not possible in any individual case to predict whether the tumor would be noninfective. For example, bird #289 carried a tumor with the external properties of noninfective tumors, yet this tumor had a high virus content.

TABLE 3.—Relation between virus-neutralizing antibody and virus content and fate of tumors: experiment 1

Group*	Bird No.	Mean diameter at time of biopsy†	Log p.f.u. per gm. of tumor	Neutralization index of serum (*7 weeks*)‡	Fate of tumor
"Noninfective"	465	10 mm. (27 days)	<1. 6	0. 95	Regressed, 10 weeks‡
	380	8 mm. (27 days)	<1. 6	0. 60	Regressed, 6 weeks
	224	9 mm. (26 days)	<1. 6	0. 86	Regressed, 7 weeks
Virus-producing tumors	334	9 mm. (28 days)	1. 8	0. 66	Regressed, 5 weeks
	450	7 mm. (28 days)	4. 9	0. 29	Progressive, died 11 weeks
	966	8 mm. (28 days)	5. 3	N.D.	Regressed, 5 weeks
	990	7 mm. (26 days)	5. 3	>1. 44	Regressed, 6 weeks
	457	9 mm. (27 days)	6. 7	0. 56	Regressed, 6 weeks
	289	6 mm. (28 days)	6. 7	1. 37	Progressive, died 22 weeks

*Tumors were selected from a larger group occurring below the 50 percent dilution endpoint, on the basis of lack of hemorrhage and firmness to palpation.

†Time given in parentheses is interval between virus inoculation and biopsy. Birds were inoculated at 10 days of age.

‡Time after virus inoculation.

It will be noted that the noninfective tumors were associated with intermediate neutralizing antibody titers. These were significantly lower than those associated with 2 birds having tumors of high virus content (#990 and 289). If high neutralizing antibody titers were the underlying cause of noninfective tumors, considerably higher levels of antibody would be expected. The observed levels associated with the noninfective tumors are in the range of 0.1 percent of the potency of hyperimmune serums. All the noninfective tumors ultimately regressed. Surprisingly, however, 4 of the 6 virus-containing tumors also regressed. Regression did not appear to be a function of neutralizing antibody content since 1 bird with relatively high levels died of progressive tumor (#289) while 2 birds with relatively low levels (#334 and 457) showed regression.

The previous experiment was, however, limited in scale and had defects in design, from the point of view of the questions asked. It would have been desirable to have obtained serum samples at the time of biopsy rather than 3 weeks later. Furthermore, an uninoculated control group

would be necessary to determine whether the neutralizing antibody in the serums of birds carrying noninfective tumors was associated with antigen liberated by these tumors. It appeared possible that this antibody was of the "natural" variety, which has been found with increasing prevalence with aging of birds from the flock used in these experiments (*18*).

A second experiment was therefore carried out to answer these questions. Ninety 10-day-old birds were inoculated subcutaneously in the wing web, with 0.2 p.f.u. per inoculum. An additional 15 birds were banded and housed in the same cages with the inoculated birds. Tumors were measured and biopsied 23 days after inoculation. Serums were drawn from all tumor-bearing and all uninoculated control birds at the time of biopsy. Five weeks after inoculation, birds carrying wing-filling hemorrhagic tumors were killed. The remaining birds were observed until obviously moribund or until tumors had completely regressed. A second serum sample was drawn from all remaining birds at 42 days after inoculation.

The results of this experiment are summarized in table 4. Titrations of virus-neutralizing activity in serums drawn 23 days after virus inoculation revealed small quantities of neutralizing material in the majority of serums in both control and experimental groups. The fact that uninoculated control serums revealed essentially identical neutralizing activity, and that this activity dropped to insignificant levels in control serums, strongly suggests that this neutralizing activity represents remaining "natural antibody" of maternal origin.

Serums drawn at 42 days revealed a significant rise in content of neutralizing antibody in the majority of those birds with virus-producing tumors. No rise was observed in the 2 birds carrying noninfective tumors or in the uninoculated controls.

In this experiment tumors were not selected for lack of hemorrhage, or firmness to palpation, as in the previous experiment. As a result, regression was seen in only 5 of 25 virus-producing tumors. The two noninfective tumors again regressed, as in the previous experiment.

The growth behavior of the noninfective tumor in bird #8 is worthy of special note. This tumor was a firm, roughly spherical mass measuring 5 mm. in diameter at the time of biopsy. Approximately half the tumor was removed for assay. When re-examined 8 days later, the tumor had increased in size to 22 $\times$ 15 mm. and was still firm and nonhemorrhagic. It was thought likely that this rapid growth represented the appearance of a virus-producing tumor. The mass was nevertheless rebiopsied since noninfective tumor was needed for tissue-culture experiments. Care was taken to remove portions from all regions of the tumor during the second biopsy. No virus was detected in extracts of this biopsy or in supernatants of tissue cultures derived from it. Nevertheless, 8 days after the second biopsy the tumor, still enlarging, measured 35 $\times$ 22 mm.

This tumor indicates that considerable growth potential may exist in noninfective tumors and, conversely, would appear to suggest that lack of

TABLE 4.—Relation between virus-neutralizing antibody and virus content and fate of tumors: experiment 2

Group*	Bird No.	Mean diameter at time of biopsy (*mm.*)†	Log p.f.u.† per gm. tumor	Neutralization index of serum		Fate of tumor
				23 days‡	42 days‡	
"Noninfective"	6	7 × 9	≤1. 6	0. 51	0. 08	Regressed, 6 weeks‡
	8	5 × 5	≤1. 6	0. 65	(+0. 16)§	Regressed, 12 weeks
				$\bar{x}$ = 0. 57	$\bar{x}$ = (+0. 12)	
Virus-producing tumors	1	6 × 7	6. 3	0. 71		Progressive, killed 5 weeks
	2	7 × 12	6. 2	0. 62	1. 55	Progressive, killed 6 weeks
	3	5 × 6	5. 7	1. 43		Progressive, killed 5 weeks
	4	11 × 12	5. 1	0. 15		Progressive, killed 5 weeks
	5	10 × 12	6. 5	0. 16	<1. 61	*Regressed*, 6 weeks
	7	40 × 14	4. 5	0. 36		Progressive, died 4 weeks
	9	20 × 15	5. 6	0. 54	0. 93	Progressive, died 12 weeks
	10	4 × 5	3. 8	0. 80		Progressive, killed 5 weeks
	11	12 × 15	6. 6	0. 41		Progressive, killed 5 weeks
	12	22 × 12	3. 7	0. 40		Progressive, killed 5 weeks
	13	7 × 10	5. 5	0. 35	0. 30	Large *stationary* tumor, 6 months
	14	36 × 14	6. 3	0. 71		Progressive, killed 5 weeks
	15	12 × 14	6. 1	N.D.	0. 45	*Regressed*, 6 weeks
	16	5 × 7	5. 5	0. 35	0. 62	*Regressed*, 8 weeks
	17	28 × 12	5. 9	0. 45		Progressive, killed 5 weeks
	18	46 × 18	6. 3	0. 48		Progressive, died 4 weeks
	19	5 × 5	4. 9	0. 33		Progressive, killed 5 weeks
	20	10 × 18	6. 3	0. 61		Progressive, killed 5 weeks
	21	26 × 14	6. 9	0. 62		Progressive, killed 5 weeks
	22	22 × 18	5. 4	0. 69		Progressive, killed 5 weeks
	23	42 × 15	7. 0	N.D.		Progressive, killed 5 weeks
	24	6 × 6	4. 2	0. 87	0. 48	Small *stationary* tumor, 6 months
	25	4 × 6	4. 9	0. 56	<1. 61	*Regressed*, 6 weeks
	26	5 × 6	5. 2	0. 36	0. 08	*Regressed*, 6 weeks
	27	8 × 10	6. 7	0. 59		Progressive, killed 5 weeks
				$\bar{x}$ = 0. 54	$\bar{x}$ = 0. 85	
Uninoculated controls	101	None		1. 23	0. 04	
	102	"		0. 46	0. 07	
	103	"		0. 07	(+0. 22)	
	104	"		0. 11	(+0. 25)	

105	"	0.39	N.D.
106	"	0.45	N.D.
107	"	0.60	(+0.20)
108	"	0.81	0.15
109	"	0.65	0.30
110	"	1.07	(+0.31)
111	"	0.93	0.48
112	"	0.65	0.61
113	"	0.62	0.51
114	"	0.81	N.D.
115	"	0.70	0.07
		$\bar{x} = 0.63$	$\bar{x} = 0.10$

* Unselected tumors after inoculation of 0.2 p.f.u. Tumor incidence was 29/90 birds inoculated.
† Twenty-three days after virus inoculation.
‡ Time after virus inoculation.
§ Neutralization indexes given in parentheses indicate that titer of neutralization mixture was higher than that of the control mixture.

virus production is not a consequence of possible mechanisms, *e.g.*, the homograft reaction, tending to limit growth of the tumor cells.

Finally, it should again be noted that there was no correlation between neutralizing antibody levels and growth or regression of tumors: *e.g.*, the tumor in bird #26 regressed in the absence of neutralizing antibody, whereas that in bird #2 progressed rapidly in the presence of high levels of such antibody.

B. Effect of inducing single-focus tumors in multiple sites per bird.—A possible means for distinguishing between cellular and host-mediated mechanisms for the genesis of noninfective tumors appeared to be inherent in the study of multiple single-focus tumors arising in birds inoculated at multiple sites. If an aberrant immune responsiveness of certain hosts was the mechanism underlying the production of noninfective tumors, it would be expected that birds giving rise to such tumors would carry only this type of tumor and, conversely, that birds carrying tumors of high virus content would carry no noninfective tumors.

Two experiments were carried out. In both experiments virus suspensions were diluted to the region of the 50 percent dilution endpoint and inoculated in 12 sites per bird. In experiment 1 (table 5) three 3- to 4-week-old birds were used. Two of three birds developed both noninfective tumors and tumors of high virus content. The over-all incidence of noninfective tumors was unusually high (6/17). The second experiment (expt. 2, table 5), in which an inoculum with half the estimated potency of that used in experiment 1 was inoculated into each of 12 sites in five 10-day-old birds, revealed a lower incidence of noninfective tumors (3/16). A wide spread in virus content of tumors was observed in birds 1 and 2. Two birds carried only tumors of high virus content and one revealed only two noninfective tumors.

TABLE 5.—Virus contents of "single-focus" tumors induced in multiple sites per bird

Experiment	Bird No.	Virus content *(log p.f.u./gm.)*: Tumor No.						
		1	2	3	4	5	6	7
1*	1	6.2	<2.3	4.6	6.3	3.0	2.8	<2.3
	2	6.0	6.3	6.7	5.5			
	3	4.9	6.6	<2.3	<2.3	<2.3	<2.3	
2†	1	6.0	5.8	4.8	2.6			
	2	<2.6	4.0	4.5	4.5			
	3	6.5	6.0	6.4	6.3			
	4	<2.3	<2.3					
	5	6.6	6.5					

*Three-to-4-week-old birds inoculated in 12 sites subcutaneously with 0.1 ml. of a 10^{-4} dilution of a virus suspension containing $10^{6.4}$ p.f.u. per ml. Tumors harvested 14 days after virus inoculation.

†Ten-day-old birds inoculated subcutaneously with 0.05 ml. of a 10^{-4} dilution of the above virus stock. Tumors harvested 13 days after virus inoculation.

The lack of a finding of a complete segregation of tumors of high and low, or nondetectable, virus content in different birds would appear to support

the hypothesis that a generalized host response does not underlie the genesis of noninfective tumors.

DISCUSSION

The demonstration by Bryan, Calnan, and Mahoney (*5*) that a proportion of tumors induced by minute doses of RSV contains no detectable virus has provided a challenging model for the study of the masking phenomenon. The generality of this phenomenon, at least with RSV, has been pointed out by Groupé and Rauscher (*6*, *19*) who have observed similar phenomena in the brains of 1-day-old chicks and in young turkeys.

A striking difference exists between the chicken and the turkey systems since in the latter "virus-free" tumors occur even when relatively large doses of virus are inoculated (*19*). The fact that turkeys become "resistant" to RSV inoculations after 3 to 4 weeks of age, and that this resistance can be markedly diminished by prior inoculation of embryos with chick blood cells (*20*), may indicate that Rous tumors induced in turkeys are subject to immune reactions which may be important in suppression of virus production in this species.

That such reactions do not underlie the genesis of noninfective tumors in 10-day-old chicks was initially suggested by the finding that they occur only as single-focus tumors, *i.e.*, those initiated by single effective virus : cell interactions.

Noninfective tumors were grossly not distinguishable from most other single-focus tumors, and their growth rates did not appear markedly different. This finding is particularly surprising when we consider the recent work by Vigier (*21*). This author correlated the growth rates of intradermal sarcomas induced by RSV and the neutralizing antibody contents of serums. He found these variables to be closely correlated except at high antibody levels where tumor growth rate approached a constant minimum. These observations were interpreted to indicate that tumor growth rate initially depended on a mixture of growth and reinfection, the extent of the latter depending on the degree of humoral immunity to virus. This conclusion would appear inconsistent with the finding that tumor growth rate is not significantly reduced in noninfective tumors. This discrepancy might be related to differences in the systems used in the two studies: *e.g.*, subcutaneous *versus* intradermal route; 10-day *versus* 1- to 2-month-old birds.

The finding that cultures derived from noninfective tumors do not produce virus, whereas those derived from virus-containing tumors do so readily, is further suggestive evidence against an immune mechanism for the genesis of these tumors. It is of interest, in this regard, that analyses of clones derived from RSV-infected fibroblast cultures revealed, in addition to virus-producing clones, a proportion of clones composed of cells which did not produce virus, but yet were resistant to reinfection (*22*).

Since similar examinations of uninfected fibroblasts revealed no resistant clones, and since resistant clones could be produced under conditions (cloning immediately after infection with identical plating efficiency in control and infected groups) in which selection did not appear to be possible, it was concluded that these clones represented a form of stable infection not productive of infectious virus. The noninfective tumors may be similarly interpreted.

The possibility that noninfective clones, or tumors, represent cells producing primarily incomplete or defective virus should be considered. Experiments have been carried out to search for particles which can "interfere" with subsequent infection by RSV, and for particles which can block the virus-neutralizing antibody (*23*). Such materials were not found in extracts of noninfective tumors. However, unfortunately, these experiments were not conclusive since ultraviolet irradiated extracts of tumors of high virus content produced interference only irregularly and possessed "serum-blocking" activity only in low dilution.

Two observations would, however, suggest that Rous tumors may consist of mixtures of varying proportions of virus-yielding and nonvirus-yielding cells. The first are those reported by Epstein (*24*), who described a relation between the number of cells in Rous ascites tumors which were associated with virus particles and the infectious virus content of these tumors. Second are the observations reported by Hagenau *et al.* (*25*). These authors studied ultrathin sections of tumors initiated by high and low doses of RSV. Within the "high-dose" group a correlation between infectious virus content and particle frequency was observed. In the "low-dose" group, 5 of 7 tumors that were examined revealed no identifiable virus-like particles.

The finding that noninfective tumors cannot be maintained on serial passage *in vivo* without return of infectious virus would be compatible with the interpretation that a small proportion of the cells of these tumors may on occasion "revert" to virus production. Indeed this may be the mechanism underlying the production of tumors of intermediate virus content.

It has been found that only a minor proportion, varying in different experiments between 0.1 and 10 percent, of cells infected with standard strains of Rous virus at any virus : cell multiplicity greater than unity will be converted into "infectious centers" (*23*, *26*). It has also been reported that these variations depend on physiologic rather than on genetic variability in the cell population (*26*). In addition clonal analyses, described previously, have indicated that a variable but major proportion of clones from infected cultures do not yield virus, yet are resistant to infection with RSV (*22*).

These findings may indicate that a relatively large proportion of single-focus tumors may originate in noninfective clones. The ultimate virus content of these tumors may then depend on the time during the development of this clone that "reversion" to virus production occurs in one or more of the cells of the clone.

The study of neutralizing antibody levels in tumor-bearing birds clearly

rules out such antibody in the genesis of noninfective tumors. These studies served also to indicate that regression is not associated with virus-neutralizing antibody. This latter conclusion is in accord with observations that hyperimmune virus-neutralizing antiserums do not affect the development of lesions on the CAM (*27*, *28*), or the minimal intrinsic growth rate of dermal sarcomas (*21*).

The finding of a high frequency of regression of single-focus tumors is in itself of interest. Friere and Duran-Reynals observed no regressions in 1,464 chicks inoculated at 15 days of age, whereas an incidence of 15 percent was observed in older birds (*29*).

It is likely that the difference between the regression incidence observed by Friere and Duran-Reynals and that observed in these experiments depends on the extremely low virus dose employed in the present experiments, since a relationship between dose and incidence of regression has been reported (*30*).

The mechanism of these regressions is unknown. The possibility that the infected cell develops new antigens, which are not of viral nature, and which cause it to be recognized as foreign by the host animal, should be considered. A similar suggestion was made by Furth *et al.* to account for the transplantation behavior of some virus-induced leukemias in mice (*31*). A homograft reaction may indeed be responsible for the genesis of the noninfective tumors. It has been reported that anticell antiserums may inhibit virus multiplication even in the absence of visible cytotoxic or growth inhibitory effects on the host cells (*32*). The study of the nature of multiple single-focus tumors in birds inoculated in multiple sites was designed to further investigate this possibility.

Since the homograft reaction might be expected to be a body-wide one, the finding of noninfective tumors in animals also carrying tumors of high virus content would suggest that this reaction is not involved. However, it is conceivable that such a reaction might be site specific, at least in its early stages. Further multisite experiments may allow a more conclusive discrimination between these hypotheses. Such discrimination would not result merely from the demonstration of a nonrandom, host-correlated incidence of noninfective tumors, since it is conceivable that different hosts have cells differing, on the average, in physiologic susceptibility to "productive" infection. However, a significant correlation between incidence of noninfective tumors and average number of tumors per bird would rule out any mechanism depending on events occurring at the time of initial infection.

Greene, in studies carried out on the masking phenomenon in the Shope papilloma virus, found that the virus content of cottontail or domestic rabbit skin infected *in vitro* did not depend on the host to which the skin was transplanted, but did depend on the species of skin employed (*33*). It was therefore concluded that the masking process depended on cellular rather than host properties.

Such a hypothesis would appear to be most compatible with the observations that have been described in the present system.

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Initiation and Promotion Activity of Certain Polynuclear Hydrocarbons[1,2,3]

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SUMMARY

When tested as initiators of carcinogenesis per 3.5 cm.² of mouse skin, 1 μg. of 7,12-dimethylbenz[*a*]anthracene was very potent, 0.1 μg. of this hydrocarbon had marginal activity, 0.9 μg. of benz[*a*]anthracene was a little more active than 0.1 μg. of 7,12-dimethylbenz[*a*]anthracene, and 9-methylanthracene was inactive. Both benz[*a*]anthracene and 9-methylanthracene were inactive as promoters of carcinogenesis.—J. Nat. Cancer Inst. 23: 1383–1387, 1959.

THE INTERPRETATIONS of the conflicting protein-binding results with benz[*a*]anthracene (*1–5*), when compared to that obtained with 7,12-dimethylbenz[*a*]anthracene, hinged upon the classification of benz[*a*]anthracene as a weak or noncarcinogenic hydrocarbon when applied repeatedly to the skin of mice (*6*). Benz[*a*]anthracene, however, has been reported to be an initiator of carcinogenesis (*7, 8*). To fill a gap in the biological aspects of these studies, the ability of benz[*a*]anthracene as an initiator of carcinogenesis was compared to that of 7,12-dimethylbenz[*a*]anthracene, under standardized conditions. The previous procedure was used (*3*), which showed that 1 μg. of 7,12-dimethylbenz[*a*]anthracene per 3.5 cm.² of mouse skin was a potent initiator of carcinogenesis. A fractionated extract of croton seeds (*9*) was used as the promoter. The potentiality of 9-methylanthracene as an initiator was also evaluated.

EXPERIMENTS

The various purified agents (*10, 11*) mentioned later were tested as initiators by the previous procedure used to demonstrate the initiator activity of 1 μg. (3.9 mμmole) of 7,12-dimethylbenz[*a*]anthracene (*3*) per 3.5 cm.² of the skin of virgin female Swiss mice in the second resting phase of the hair cycle. The test for a promoter was conducted by

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[3] An abstract of some of this work appeared in Proc. Am. Assoc. Cancer Res. 2: 113, 1956; 2: 304, 1958.

substituting an acetone solution of the agent being tested for the fraction of croton seeds [DJ-14 (*9*)][4] in the previous procedure. The test for a complete carcinogen was conducted by the twice weekly application of an acetone (distilled, fluorescence-free) solution of the compound under test. The agents tested as initiators per 3.5 cm.^{2} of mouse skin were: (*a*) 0.1 μg. (0.39 mμmole) of 7,12-dimethylbenz[*a*]anthracene, (*b*) 0.9 μg. (3.9 mμmole) of benz[*a*]anthracene, (*c*) 0.75 μg. (3.9 mμmole) of 9-methylanthracene. The tests for promoters and complete carcinogens were carried out with an 0.27 percent solution of benz[*a*]anthracene and an 0.23 percent solution of 9-methylanthracene. Data more extensive than previously published (*3*) were obtained with both 1 μg. (3.9 mμmole) of 7,12-dimethylbenz[*a*]anthracene as an initiator per 3.5 cm.^{2} of mouse skin and with DJ-14 alone. The same batch of DJ-14 was used in all experiments in this report. The identification of tumors by gross examination was occasionally confirmed by microscopic examination.[5]

RESULTS AND DISCUSSION

In these studies the initiating effects of single doses of the hydrocarbons were evaluated by using a fractionated extract of DJ-14 as a promoting agent (*9*). This procedure was dependent on the noncarcinogenicity of the promoter. The possible carcinogenicity of croton oil has been recently suggested (*12, 13*) and discussed (*14*). The data (tables 1 and 2) show that DJ-14 was useful as a promoter in these experiments even though papillomas were observed, without initiation.

It was evident (tables 2 and 3) that 1 μg. (3.9 mμmole) of 7,12-dimethylbenz[*a*]anthracene was a much more potent initiator of carcinogenesis than either 0.9 μg. (3.9 mμmole) of benz[*a*]anthracene or 0.1 μg. (0.39 mμmole) of 7,12-dimethylbenz[*a*]anthracene.[6] Also 0.75 μg. (3.9 mμmole) of 9-methylanthracene showed no activity as an initiator of carcinogenesis. Thus the similar binding results with 7,12-dimethylbenz[*a*]anthracene (*3*) and benz[*a*]anthracene (*5*) and the binding with 9-methylanthracene (*4*) found in this laboratory were not directly related to the initiator activity of these compounds.

On the basis of the yield of tumor-bearing animals, 0.9 μg. (3.9 mμmole) of benz[*a*]anthracene has been classified as a slightly more potent initiator than 0.1 μg. (0.39 mμmole) of 7,12-dimethylbenz[*a*]anthracene per 3.5 cm.^{2} of mouse skin. Also this latter dose of 7,12-dimethylbenz[*a*]anthracene represented a marginal response as an initiator of carcinogenesis under the test conditions described.

The results obtained with benz[*a*]anthracene confirmed the previous reports that benz[*a*]anthracene was an initiator. Relatively large amounts

[4] Supplied by Dr. Jean Sicé.

[5] Dr. Philippe Shubik provided the microscopic examination. Miss Francine Cioffi tabulated the data.

[6] These results with 7,12-dimethylbenz[*a*]anthracene conformed to the findings of Klein (*15*) who reported that 0.4 μg. was a decided initiator and 0.16 μg. was a questionable initiator. When calculated on a basis of 3.5 cm.^{2} of skin, these doses were about 1.2 μg. and 0.47 μg. respectively. Klein used croton oil as the promoter.

TABLE 1.—Tumor incidence during the repeated application of various substances on mice*†

Initiator	Duration of treatment (*weeks*)									
	0	6	10	15	20	25	30	40	50	60
DJ-14‡	0/0(20)	0/0(19)	0/0(19)	1/1(19)	0/0(19)	0/0(19)	0/0(18)	5/2(17)	4/2(17)	5/2(9)
DJ-14‡ §	0/0(20)	0/0(20)	0/0(20)	0/0(20)	0/0(20)	1/1(20)	4/3(20)	5/3(17)	3/2(13)	1/1(9)
Benz[*a*]anthracene‖	0/0(20)	0/0(20)	0/0(20)	0/0(20)	0/0(20)	0/0(20)	0/0(19)	0/0(19)	0/0(19)	0/0(15)
9-Methylanthracene¶	0/0(20)	0/0(20)	0/0(19)	0/0(19)	0/0(19)	0/0(19)	0/0(19)	0/0(18)	0/0(15)	0/0(15)

*A drop of about 0.02 ml. of solutions of the various substances dissolved in acetone was applied twice each week.

†The results are expressed as the number of tumors that persisted for at least 3 weeks, divided by the number of tumor-bearing animals, and include tumors of animals that died while bearing tumors. Tumors that regressed are not included. The number of surviving animals is in parentheses.

‡The concentration of the solution was 0.5 percent. Most of the tumors were papillomas.

§This experiment was begun 6 months after the start of the previous experiment. One skin carcinoma was observed.

‖The concentration of the solution was 0.27 percent. Tumor-bearing animals appeared in the 83d and 89th weeks. Both tumors were skin carcinomas.

¶The concentration of the solution was 0.23 percent.

TABLE 2.—Several polynuclear hydrocarbons tested as initiators of carcinogenesis* †

Initiator	Duration of treatment (*weeks*)									
	0	6	10	15	20	25	30	40	50	60
1 μg. of 7,12-Dimethylbenz[*a*]anthracene	0/0(20)	2/2(20)	19/13(20)	28/16(20)	24/13(18)	13/10(18)	18/12(18)	11/7(18)	17/10(16)	14/10(11)
0.1 μg. of 7,12-Dimethylbenz[*a*]anthracene	0/0(20)	0/0(20)	0/0(20)	1/1(20)	7/5(20)	7/5(20)	5/3(20)	9/5(17)	9/5(13)	8/4(10)
0.9 μg. of Benz[*a*]anthracene	0/0(20)	0/0(20)	0/0(20)	1/1(20)	1/1(20)	1/1(20)	4/4(20)	10/8(18)	10/8(16)	14/8(10)
0.75 μg. of 9-Methylanthracene	0/0(20)	0/0(20)	0/0(20)	0/0(20)	0/0(20)	2/2(20)	0/0(20)	3/2(17)	3/2(15)	3/2(8)

*The designated amount of materialwas dissolved in 50 μl. of an acetone solution which was applied once over 3.5 cm.² of mouse skin. Twice weekly treatment with drops approximately 0.2 ml. of an acetone solution (0.5%) of JD-14 was begun 1 week later and represented zero time of the experiment.

†The results are expressed as the number of tumors that persisted for at least 3 weeks, divided by the number of tumor-bearing animals, and include the tumors of animals that died while bearing tumors. Tumors that regressed are not included. The number of surviving animals is in parentheses. Most of the tumors were papillomas with a few skin carcinoms.

TABLE 3.—Carcinogenicity of single application of several polynuclear hydrocarbons*†

Application	Time (*weeks*)			
	0	10	40	60
7,12-Dimethylbenz[*a*]anthracene (1 μg.)	0/20	0/20	0/19	0/17
Benz[*a*]anthracene (0.9 μg.)	0/20	0/20	0/19	0/16
9-Methylanthracene (0.75 μg.)	0/18	0/17	0/15	0/9

*The material was dissolved in 50 μl. of an acetone solution which was applied once over 3.5 cm.² of mouse skin. There was no further treatment.

†The results are expressed as tumor-bearing animals divided by surviving animals.

of this hydrocarbon were used in the earlier experiments. Graffi and coworkers (*7*) applied one drop of an acetone solution (0.5%) of the hydrocarbon alternately with weekly applications of croton oil, in mineral oil, while Roe and Salaman (*8*) applied 3 mg. of benz[*a*]anthracene, dissolved in acetone, to mice twice in 2 successive weeks before the repeated applications of croton oil, dissolved in acetone, were begun. The present findings dispelled the possibility that a trace impurity in benz[*a*]anthracene was responsible for the initiator activity of this compound.

When tested by the repeated application on the skin of the mouse (table 1), the tumor response [7] with benz[*a*]anthracene was extremely low, as observed by other investigators (*6*). The response with 9-methylanthracene was negative, in agreement with previous reports (*17*).

Tumors were not elicited when benz[*a*]anthracene and 9-methylanthracene were tested as promoters (table 4).

TABLE 4.—Test of benz[*a*]anthracene and 9-methylanthracene as promoters

	Duration of treatment (*weeks*)*†			
	0	10	40	60
Benz[*a*]anthracene‡	0/19	0/18	0/11	0/5
9-Methylanthracene§	0/21	0/21	0/19	0/12

*The mice were painted once with 50 μl. of an acetone solution containing 1 μg. of 7,12-dimethylbenz[*a*]anthracene. The treated area was 3.5 cm.² of skin. Twice weekly treatment with drops of approximately 0.02 ml. of solutions of the various substances dissolved in acetone was begun 1 week later and represented zero time of the experiment.

†The results are expressed as tumor-bearing animals per surviving animals.

‡The concentration of the solution was 0.267 percent.

§The concentration of the solution was 0.225 percent.

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Effect of Bone Marrow Culture *In Vitro* on Its Protective Action in Irradiated Mice [1, 2]

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SUMMARY

Mouse bone marrow cells tissue-cultured in a special container have been studied in an attempt to identify the cells responsible for the protection afforded recipient, lethally irradiated mice, by bone marrow post treatment. After 4 days of culture, a decreased protective activity of injected tissue-cultured cells was observed. By the 9th day of life *in vitro*, the explanted bone marrow cells had essentially lost their protective capacity. Analysis of the cell species present at this time showed that several stem-cell types persisted in such negative cultures. These findings are discussed in relation to the mechanism of protection of recipient mice, with bone marrow.—J. Nat. Cancer Inst. 23: 1389–1395, 1959.

THE SPECIFIC nature of the donor tissues required for protection of irradiated mammals suggests an involvement of a special cell species in the modification of irradiation injury. Nonhemic tissues and cell-free fractions from active cells have been shown by various investigators to influence mortality or other definitive endpoints. At levels of ionizing radiations sufficient to kill all the animals, however, only living cell preparations from hematopoietic tissues prevent acute radiation-induced death. From the available information, it is clear that not all the cells present in spleen, bone marrow, or fetal liver are necessary for recovery. The terminal cells of the erythropoietic and myeloid series may be ruled out by the negative results generally observed after treatment of exposed mice, with large quantities of cells from the peripheral blood (*1*). With minor exception, cells derived from the thymus and lymphocytes have been shown to be without protective activity (*2*). Ground bone has been reported effective in enhancing survival of recipient irradiated mice (*3*). The contamination of the bone fragments with hemic elements makes interpretation of this finding difficult. Lorenz and Congdon reported a 50 percent survival in otherwise lethally irradiated animals receiving a

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cell suspension of a reticulum-cell sarcoma (*2*). These results were equivocal. Cole and coworkers (*4*) induced hypertrophy of erythropoietic tissue by prior phenylhydrazine treatment, with no enhancement of the protective effect of splenic homogenates against whole-body irradiation. These investigators also obtained relatively pure suspensions of monocytes from the peritoneal fluid of rats and observed a lack of protection in recipient mice.

With the tissue-culture method, our laboratory has studied bone marrow cell population changes during cultivation *in vitro* in an effort to acquire further information on the mechanism whereby recovery is brought about in recipient exposed mice. The results of studies with a vaccine-vial method of bone marrow culture have been reported (*5*). A preliminary report has been made (*6*) on the protection afforded by altered bone marrow cell populations during the several phases of development observed *in vitro*, with the use of a *well* method developed by Reisner (*7*). Additional details and results are described herein.

MATERIALS AND METHODS

Bone marrow procurement and culture.—Bone marrow plugs were obtained from the femurs of 9- to 15-week-old (C3H $\times$ 101)F_1 hybrid mice and explanted as 1- to 2-mm. fragments into a glass well fixed onto the surface of a slide, as described in detail in a previous publication (*6*). The medium used consisted of 80 percent mixture 199 supplemented with 20 percent horse serum (Microbiological Associates), plus 50 μg. of streptomycin and 80 units of penicillin per ml. Feeding schedules were related to *p*H change. When the phenol-red indicator assumed a yellowish appearance, approximately one half the old medium was removed and replaced with fresh medium. As might be expected, this change in *p*H was most rapid in young cultures (every 24–48 hours), slowing with age as the number of metabolizing cells decreased.

Harvesting of cells.—The cells were detached from the glass surface, after a short period of trypsin exposure, by means of a rubber policeman. Many cells were undoubtedly destroyed in this step, but because of a number of technical factors such as the need to harvest the largest number of cells possible without unnecessary exposure to adverse chemicals or enzymes for prolonged periods, this procedure has been routinely followed. The harvested cells were diluted in cold medium and harvested as quickly as possible in a Servall refrigerated centrifuge at 1,000 rpm (121 $\times$ g) for 12 minutes. The pellet of cells was then resuspended in a limited volume of fresh medium and cell counts were made with a hemocytometer, in the presence of eosin. Only eosin negative cells were counted. In fresh bone marrow preparations, 98 percent of the nucleated cells present were viable by the test. All cell counts listed refer to these nonstaining cells. The suspension was then diluted further to the desired final concentration for intravenous tail-vein injection. Since the number

of cells *in vitro* dropped off sharply, it was difficult after the first 48 hours to harvest an optimal number of cells from cultures for testing. The number of recipient animals in a given experiment was thus limited by the supply of harvested cells.

Preparation of slides and cell identification.—Thin smears of concentrated cells were air dried rapidly, fixed in methyl alcohol overnight, and stained with a combination Giemsa-Wright's stain. A differential count of a portion of at least two slides per sample was made for the myelogram data. Only cells with visible cytoplasm in addition to nuclei were counted. Other investigators have reported and discussed the difficulties encountered in identifying the various cell types found in mouse and rat marrow (*8–10*). We confirm this difficulty and emphasize the subjective nature of such analysis.

Irradiation of recipient mice.—All mice used for assay of the bone marrow preparations were (C3H × 101)F_1 hybrids exposed to 900 r of X rays. The details of X-ray exposure are published elsewhere (*6*). Experiments were limited to 12- to 15-week-old mice. Earlier studies had shown that 0.5 to 1.0 × 10^6 fresh bone marrow cells would consistently protect mice in this age range exposed to 900 r (*11*). It was subsequently found that younger mice required larger doses of fresh bone marrow for equivalent protection at 900 r (Billen: unpublished observations).

RESULTS

As shown previously (*6*) the ability of tissue-cultured bone marrow cells to protect recipient irradiated mice was limited to the first several days of life *in vitro*. From the results of several additional experiments it has been established that beyond 4 days the effectiveness of the cells steadily declines (table 1). By the 9th day of culture the protective potential of the cell population was essentially nil. In duplicate experiments there was some variation from the results presented in table 1, but the general trend was similar. The establishment of age limits for effective *versus* noneffective cultures becomes important in the evaluation of the relationship between bone marrow cell species and protection.

TABLE 1.—Modification of X-ray effects by tissue-cultured bone marrow cells

Experiment No.	Age of culture (*days*)	Number of cells injected per animal (*millions*)	Thirty-day survivors	Survivors (*percent*)
			No. tested	
1	4	3.6	12/12	100
2	6	3.0	4/9	44
3	6	3.2	3/8	38
4	6	3.6	2/11	18
5	7	2.0	3/11	27
6	9	2.0	0/9	0
7	9	3.0	1/5	20
8	13	1.2	0/5	0
All controls	—	—	0/65	0

Table 2 presents the results of differential counts made on stained smears of cells from representative cultures. In general, the classification of the several cell species found in rat bone marrow and described by Harris and Burke (*11*) has been adopted here because of the striking morphological similarities between the rat and the mouse cells. The photographs shown by these investigators of the various cell species of the rat marrow could easily be substituted for those found in mouse bone marrow. The distribution of bone marrow cell species in (C3H × 101)F_1 mice is similar to that reported for other strains of mice (*9*). The characteristic shift *in vitro* in population (*12*) from one composed primarily of myeloid and erythroid elements to one composed mostly of monocytes, macrophages, and fibroblasts is seen from the data presented in table 2. There is a high level of granulocytes observed for several days after explantation. This is probably accounted for by a lifespan of several days for existing granulocytes and the formation of additional mature cells from myelocytic precursors. As the granulocytes degenerate they are not replaced in sufficient numbers. This results in a drastic drop in cell count during the first 7 to 10 days of culture.

TABLE 2.—Myelogram of bone marrow of (C3H × 101)F_1 mice

Classification	Percent distribution		
	Fresh preparation (556)*	Four-day culture (1,099)*	Nine-day culture (495)*
Mature granulocytes	56	69	15
Myeloblasts and myelocytes	14	10	6
Normoblasts	15	<1	<1
Megakaryocytes	<1	<1	0
Lymphocyte-like cells	13	15	10
Miscellaneous:			
Macrophages, monocytes, fibroblasts, unclassified	2	6	69

*Number of cells counted.

Mitotic figures were seen throughout this period but always in an extremely low number, less than 0.1 percent of the cells studied in stained preparations. In the 9-day cultures, mitotic figures were still observed in cells identified as myeloblasts or myelocytes by the presence of granules and other morphologic criteria (fig. 6). The presence of myelocytes was confirmed by time-lapse phase cinematography (*6*). The myelocytic series was also observed in 18-day cultures (*6*). The use of horse serum in the medium proved detrimental to erythropoietic elements. Either the hemagglutinins found in the horse serum or other unidentified factors contributed to a marked decline in red cell elements shortly after suspension of the bone marrow cells in the medium. By the 4th day it was difficult to find and make a positive identification of erythroid cells (table 2).

DISCUSSION

The presence of cells morphologically resembling stem-cell types (lymphocyte-like and myeloblasts, figs. 1 through 6) in cultures no longer exhibiting protective activity in recipient irradiated animals was surprising, in light of the massive evidence suggesting a repopulation of the irradiation-depleted hemic system by donor cells. The lack of protection by populations of tissue-cultured cells containing differentiated cells still capable of division may be explained by (1) an insufficient quantity of seeding cells; (2) alterations in the specific functions or potentials of these cells, which result in a loss of ability to serve as precursors of the several terminal blood elements; (3) failure of such cells to multiply on introduction into the irradiated host, and (4) the absence of a specific cell type found only in fresh bone marrow or cultures of short duration *in vitro* and not sufficiently different morphologically from other primitive blood cells to be identified.

The possibility that insufficient numbers of stem cells were administered is pertinent and deserves further consideration at this time. Dose experiments had shown that 300,000 or more nucleated cells, injected intravenously into 12- to 15-week-old (C3H $\times$ 101)F_1 mice exposed to 900 r, would consistently provide good protection (*11*). The use of the dye-uptake method as a means of differentiating dead from viable cells in arriving at the quantity of living cells injected, in this study, is far from satisfactory. For example, resistance to dye penetration and capacity to divide cannot be equated. However, eosin-negative cells usually appear in phase-contrast photography to be intact and capable of exhibiting motion and other activities usually observed in normal living preparations. With these reservations in mind we will assume eosin-negative cells to be viable, in large part.

If we assume roughly one third of the cells in fresh bone marrow to be stem cells (not including late normoblast phase cells), then 100,000 to 200,000 of such cells are capable of bringing about almost complete recovery in recipient exposed mice. If 10 to 15 percent of the cells in 9-day cultures (table 2) are hemic-precursors, then in an administered dose of 2,000,000 cells there are at least 200,000 such cells. In terms of fresh bone marrow, the number of stem-cell types in the 9-day cultures should have been sufficient to protect the recipient mice.

If the above analysis is correct, then the negative character of older cultures would be due to one of the alternative possibilities presented. Determining the identification of various blood elements as either donor- or host-derived may provide information on the potential *in vivo* of injected tissue-cultured bone marrow cells.

Two additional comments on the bone marrow cell species involved in protection are pertinent. As seen in table 2, very few erythropoietic elements remain in protective 4-day cultures. This does not necessarily rule out the erythroid elements as necessary for recovery of recipient irradiated animals, since the primitive cells may still be capable of pro-

viding such elements on transplantation into the host. In future studies we hope to determine by immunological methods the derivation of erythrocytes in host mice protected with 4-day cultures. The absence of megakaryoblasts or megakaryocytes in cultures maintained for a week or longer raises the question of the importance of these platelet precursors in bone marrow protection.

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Plate 139

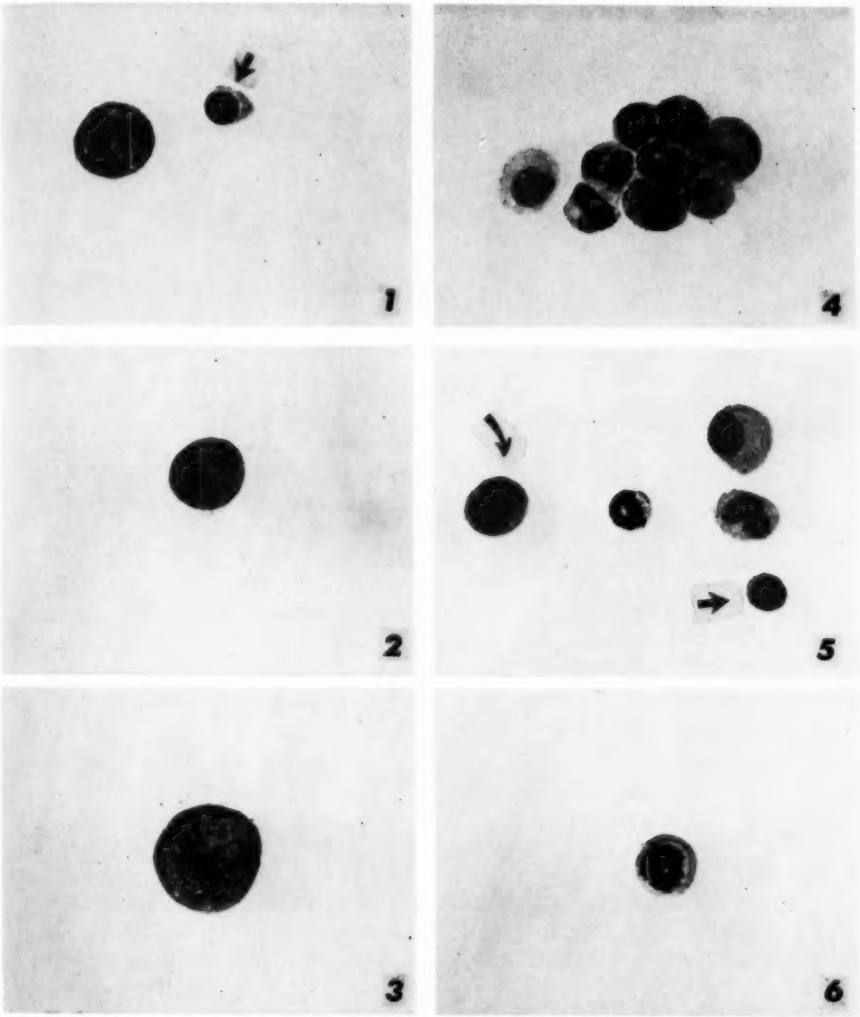
Representative hemic cells from a 9-day bone marrow tissue culture.

Figures 1 to 3.—Lymphocyte-like cell (*arrow*) and myelocytes in several stages of development.

Figure 4.—Clump of myelocytes.

Figure 5.—Lymphocyte-like cell (*straight arrow*), myeloblast (*curved arrow*), and monocytes.

Figure 6.—Myelocyte in mitosis.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Mortality in Acute Lymphocytic Leukemia [1,2]

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SUMMARY

The survival of 106 patients treated for acute lymphocytic leukemia during the period 1948–58 was analyzed by the modified life-table technique. The median survival was 4.5 months from the time of diagnosis and 6.5 months from the time of onset. There was a prolongation of approximately 8 weeks in the median survival of these leukemic individuals when compared with the median survival of an earlier group (1913–47). However, the prolongation of life in the more recent study group is of such a modest nature that it becomes demonstrable only with the aid of elaborated biostatistical techniques.—J. Nat. Cancer Inst. 23: 1397–1401, 1959.

THE MORTALITY data of 149 patients with acute lymphocytic leukemia observed at the University of California Hospital, San Francisco, during the period 1913–47, have already been reported (*1*). In this report we analyze the survival experience of the 106 patients with the same disease, who attended this Institution during the 11-year period from 1948 to 1958.

DEFINITION, MATERIAL, AND METHOD

Included in this study are all the patients for whom a diagnosis of acute lymphocytic leukemia was established at the University of California Hospital during the period from January 1, 1948, to December 31, 1958. Every clinical record was personally examined, and in all but 2 cases, diagnosed at autopsy, the clinical diagnosis had been substantiated by the hematologic findings in the peripheral blood and bone marrow. In 3 instances the presence of lymphosarcoma had been histologically documented from 1 to 5 months before the diagnosis of acute lymphocytic leukemia was made. Twice the acute leukemic process developed in patients with a long-standing anemia of obscure origin.

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[2] Assisted by a grant from Mr. and Mrs. W. S. Cowgill, in memory of Charles A. Christenson.
[3] Medical Student Fellow of the National Foundation.

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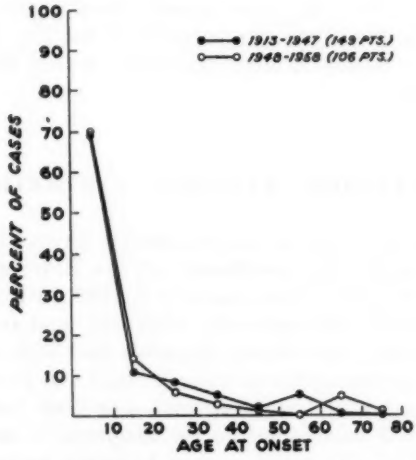
There were 106 patients with acute lymphocytic leukemia available for study, of which 57 were males and 49 females. Five patients were lost to follow-up, 5 were still alive on June 30, 1959, and an autopsy had been performed on 57 of the 96 patients whose deaths had been ascertained.

In the original communication (*1*) Shimkin determined the duration of the disease from its apparent clinical onset to death or to the latest follow-up prior to the initiation of the study. He specified that if there was a question as to whether the symptoms could have been due to leukemia, or if such symptoms were not continuous and progressive until the diagnosis was established, the date of diagnosis was taken as the date of onset. The same criteria were adopted in the present study, and the status of the patients was determined as of June 30, 1959. The survival rates were computed by means of the actuarial or modified life-table method (*2*). Differences between groups were analyzed according to the method outlined by Merrell and Shulman (*3*).

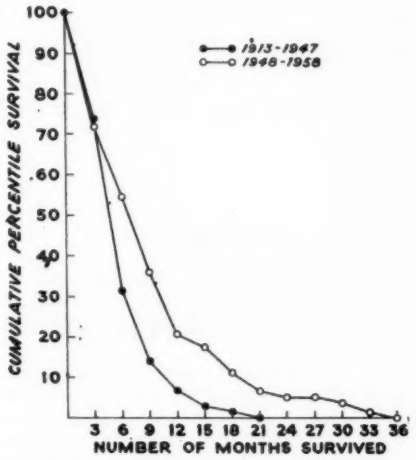
RESULTS

Text-figure 1 presents the age distribution of the subjects who form the basis of this analysis and of those incorporated in the 1913–47 study. The age distribution is almost identical for the two groups. The male-female ratio in the earlier group was 1.6 compared to a ratio of 1.2 for the present series. Neither group demonstrated a significant difference in the observed mortality between males and females.



TEXT-FIGURE 1.—Percentile distribution of cases of acute lymphocytic leukemia, by age of patient at onset of disease: University of California Hospital, San Francisco; two chronologic periods.

Text-figure 2 summarizes graphically the survival experience of the 1948–58 group compared with the 1913–47 group reported previously (*1*). The median survival in the more recent group (1948–58) is 6½ months, or about 8 weeks longer than the survival of the group (1913–47) previously reported. The earlier group (1913–47) displays a somewhat faster rate of depletion than the more recent one (1948–58); however, differences are present only among subjects surviving the first 3 months of their illness. Differences in cumulative probabilities of surviving 6, 9, 12, 15, and 18 months are significant (for the first 2 endpoints $P<0.01$; for the other 3 endpoints $P<0.05$).

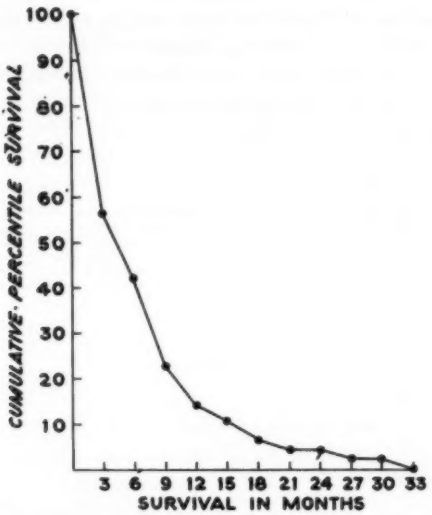


TEXT-FIGURE 2.—Cumulative percentile survival from date of onset of disease, both sexes combined: University of California Hospital, San Francisco; two chronologic periods.

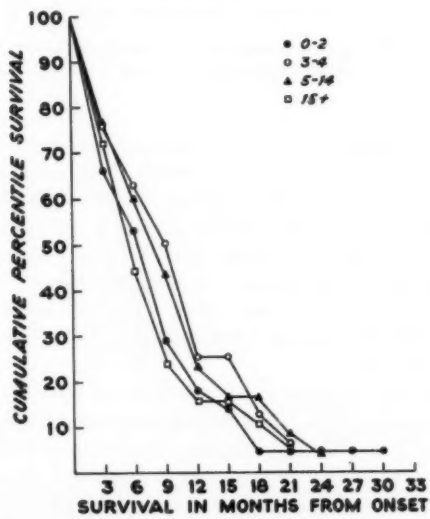
These findings suggest that the observed increase in median survival, though modest, is a real one. In addition, these data indicate that the mortality rate of the more recent group was not influenced if one considers either those individuals whose illness is so acute that they die within 3 months of onset, or those who are able to survive 1½ years, with acute lymphocytic leukemia. No patient was known to have survived more than 36 months after onset. For the same group, survival from diagnosis did not exceed 33 months, with a median survival of 4½ months (text-fig. 3).

An analysis of the longevity by the age of patient at onset of the leukemia suggests that a greater survival was experienced by individuals in age groups 3 to 4 and 5 to 14 than by those under and over these ages (text-fig. 4). The median survival was 6.5 months for ages 0 to 2; 9.0 months for ages 3 to 4; 7.5 months for ages 5 to 14; and 5.5 months for the individuals over 14 years of age. A similar age-linked trend in survival by broad age groups was reported earlier from the University of

California (*1*) and was manifested also in the patients reported by Burchenal (*4*). In Burchenal's series of patients seen after 1952, the median survival was 12.4 months for children and approximately half that time for adults.



TEXT-FIGURE 3.—Cumulative percentile survival from diagnosis, both sexes combined: University of California Hospital, San Francisco, 1948–58.



TEXT-FIGURE 4.—Cumulative percentile survival by age of patient at onset of disease, both sexes combined: University of California Hospital, San Francisco, 1948–58.

We have no explanation for the difference in survival between the observation of Burchenal and our own. Moreover, acute lymphocytic leukemia runs a slightly less rapid course than its myeloid counterpart (*5*). This observation accentuates the difference between the median survival observed here and the one reported by Burchenal inasmuch as his data were not limited to subjects with lymphocytic leukemia. It is also noteworthy that although acute leukemia in general attacks males more frequently than females (*6*), a sex difference in survival was not present in our group of acute lymphocytic leukemia subjects. This is in accord with observations made by other investigators (*1*, *5*, *7*) and might indicate that the factors which condition the development of acute leukemia might differ from those which determine its subsequent course. This observation calls to mind a situation exemplified by the lack of demonstrable human carcinogenicity of certain endocrine secretions which, when properly manipulated, may effectively alter the growth of an existing cancer (*8*, *9*).

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Delayed Mortality of Radiation Chimeras: A Pathological and Hematological Study [1]

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SUMMARY

The hematological and pathological changes in lethally X-irradiated mice treated with isologous, homologous, or heterologous (rat) bone marrow are reported. Two different disease entities were observed in mice treated with homologous or heterologous bone marrow. Bone marrow aplasia chiefly occurred before the 30th day after irradiation. This resulted in death from septicemia or hemorrhage. The secondary bone marrow failure is explained by an immunological reaction of the host against the bone marrow graft. After the 30th postirradiation day, the animals were emaciated and had dermatitis, pneumonia, diarrhea caused by focal colitis with crypt degeneration, and necrosis of the liver. The delayed foreign bone marrow reaction is of a complex nature. Impaired recovery of radiation-induced lesions, decreased resistance to bacterial infections, probably due to generalized atrophy of the lymphatic tissues, as well as an immunological reaction of the graft against the host are postulated to be causal factors. An important difference was noted between animals treated with heterologous and those treated with homologous bone marrow. Bone marrow aplasia predominated in the mice of the former group. In contrast, the mice of the latter group were chiefly affected by pneumonia, diarrhea, and colitis. Regeneration of the lymphatic tissues occurred within a month after irradiation in animals treated with isologous bone marrow, but it was retarded or failed to occur in animals treated with foreign bone marrow. The possible relation of the lymphatic-tissue changes to the etiology of the "secondary disease" is discussed.—J. Nat. Cancer Inst. 23: 1403–1439, 1959.

ACUTE MORTALITY after lethal doses of total-body X radiation may be prevented by bone marrow transplantation. The therapeutic effect is due to the repopulation of the hematopoietic tissues of the irradiated host by hematopoietic cells derived from the administered bone marrow (*1–5*).

By treating irradiated mice with isologous bone marrow, 100 percent survival can be attained (*6*). In contrast, after treating them with homologous or heterologous bone marrow, delayed mortality occurs

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[2] The authors acknowledge the continued help and valuable advice of Dr. D. W. van Bekkum, and are indebted to Miss L. Budke, Miss L. Vermeulen, and Miss F. Lubbe for their skilled technical assistance.

(*7–10*). According to Congdon and Lorenz (*8*) the delayed mortality is accompanied by bacterial infections. Other workers have reported the occurrence of a peculiar syndrome in the 2d and 3d month after irradiation, which is characterized by weight loss, diarrhea, and skin changes (*11–13*). Obviously, examination of the peripheral blood and the blood-forming organs seemed to be necessary to determine the cause of a disease which follows bone marrow transplantation.

Hematological examination of the peripheral blood of irradiated mice treated with bone marrow has been performed by several workers (*8*, *9*, *14–16*). These studies were mainly concerned with regeneration of the peripheral blood cells, and have not been extended beyond the 30th day after irradiation.

Trentin (*17*) reported that irradiated mice treated with homologous bone marrow showed a high mortality between the 21st and 60th days after irradiation, and that normal values were found for erythrocytes, leukocytes, and thrombocytes. This strongly suggested that the mortality is not caused by bone marrow aplasia, but is due to factors other than mere radiation damage. Moreover this author observed that mice treated with isologous, F_1 hybrid, and homologous bone marrow showed a relative excess of granulocytes over lymphocytes, though the period in which this abnormality was observed is not mentioned.

Regeneration of bone marrow, lymph nodes, and thymus after bone marrow or spleen transplantation has been studied by several authors (*3*, *8*, *9*, *18–20*). Pathological changes in radiation chimeras have been described by Denko (*21*), Congdon (*22*), Ilbery *et al.* (*23*), and Congdon, Makinodan, and Gengozian (*24*). These investigators have given conflicting interpretations which do not point to a single cause of the secondary mortality. The results of these workers have been partly corroborated and extended further by the present investigation, which was intended to clarify the cause of delayed mortality in radiation chimeras.

To unravel the possible complex etiology of secondary mortality, a comparison of normal with irradiated mice and of untreated control mice with irradiated mice treated with isologous or foreign bone marrow seemed to be imperative.[3] Furthermore, the incidence of the pathological and hematological changes had to be correlated closely with the time of mortality, the weight curves, and the symptoms of mice with the so-called secondary disease. In principle, changes in irradiated mice treated with bone marrow might be due to radiation-induced damage, the presence of a foreign graft of hematopoietic tissues, or both factors.

MATERIALS AND METHODS

Animals.—The irradiated bone marrow recipients were CBA males 10 to 12 weeks of age, weighing 20 to 25 gm. Female CBA and C57BL

[3] In the following presentation "treatment with foreign bone marrow" designates treatment with homologous or heterologous (rat) bone marrow.

mice 6 to 8 weeks of age were used as donors of the isologous and homologous bone marrow, respectively. Both the C57BL and the CBA strains were obtained in 1953 from Dr. O. Mühlbock, Antoni van Leeuwenhoekhuis, Amsterdam, and have since been maintained by strict brother × sister inbreeding. Young females of an inbred Wistar albino strain (WAG), 6 to 8 weeks of age and weighing 100 to 120 gm., were used as donors of rat bone marrow.

The mice were kept 5 to a cage and had access *ad libitum* to a commercial cubed food. Details on the pre- and postirradiation sanitation of the animals have been presented elsewhere (*25*).

Irradiation.—The mice were irradiated in groups of 10 or 15 in a circular perspex cage. The physical constants of the X irradiation were: 200 kv., 20 ma., filtration 1.5 mm. of Cu, HVL, 1.8 mm. of Cu, distance to target 50 cm., dose rate 45 r per minute. The dosage was measured on the bottom of the perspex box with a Baldwin substandard ionization chamber. Maximal backscatter was used.

The mice were irradiated with 675 r (LD100/30). The relation between X-ray dose and mortality has been published previously (*13*).

Bone marrow suspensions.—The suspensions were prepared by squeezing the mashed tissue through nylon-gauze filters. Bone marrow was obtained from the femurs of mice and rats. A hemocytometer was used to estimate the number of viable, nucleated cells in Tyrode's solution containing 0.2 percent eosin. The cells that stained with eosin were considered dead.

The cell suspensions were injected into the tail vein in a volume of 0.5 ml. within 4 hours after irradiation.

The number of viable, nucleated bone marrow cells administered was 6.4×10^6 for the isologous, 22.5×10^6 for the homologous, and 50×10^6 for the heterologous (rat) bone marrow.

Hematological technique.—In each blood sample the number of erythrocytes, reticulocytes, thrombocytes, and leukocytes, as well as the hemoglobin content, was estimated. In addition a differential count of the leukocytes was performed. The blood cells were enumerated in a hemocytometer. The erythrocytes were counted after dilution in Dacie's fluid (*26*), the leukocytes, after dilution in a 2 percent solution of phosphoric acid in distilled water. The thrombocytes and the reticulocytes were counted according to the method of Cazal and Izarn (*27*).

The hemoglobin content was estimated with the alkaline hematin method in a Beckman-DU spectrophotometer. A differential count of the leukocytes was obtained from May-Grünwald-Giemsa stained smears. The methods for identification of rat and mouse erythrocytes and rat granulocytes have been published previously (*6*).

In the interpretation of the hematological results, values for normal CBA males were used which were previously estimated with the aid of identical techniques. Values which differed more than 2 standard deviations from the mean were considered abnormal.

Autopsies and histological technique.—Animals killed or that died were

autopsied. Tissues for microscopic examination were obtained from those animals in which postmortal autolysis had not proceeded too far. The tissues were fixed in Bouin's fluid and embedded in paraffin. Sections of 7 μ were made and stained with hematoxylin and eosin. Smears of femoral bone marrow were stained according to the May-Grünwald-Giemsa technique. Some of the sections were stained with the periodic acid-Schiff reaction, the von Kóssa method for calcium, and the turnbull blue method for ferrous iron.

Experimental design.—Male CBA mice were irradiated with 675 r (LD 100/30). Twenty-five were used as irradiated, untreated controls, 53 were treated with isologous, 120 with homologous (C57BL), and 120 with heterologous (rat) bone marrow.

The unirradiated, untreated control group consisted of 40 CBA males of the same age as the irradiated mice. At weekly intervals the animals were weighed and inspected for the occurrence of diarrhea. Regularly mice were selected at random for examination. Blood samples were taken, the mice were killed and autopsied, and tissues were taken for microscopic examination. The hematological data of the peripheral blood were therefore obtained from a limited number of experimental animals. In addition mice treated with homologous and heterologous bone marrow and that died spontaneously were autopsied. Since the important lesions, *viz.*, bone marrow aplasia, colitis, liver necrosis, atrophy of the lymphatic tissues, vasculitis, bleeding tendency, and septicemia, were found in the mice that were killed, as well as in those that died spontaneously, the pathological data of both groups will be presented together.

An autopsy was performed on 15 unirradiated, untreated controls, 25 irradiated, untreated controls, 39 animals treated with isologous bone marrow, 109 treated with homologous bone marrow, and 94 treated with heterologous bone marrow. Tissues of the 40 control animals, the 39 animals treated with isologous bone marrow, and of 59 animals treated with homologous bone marrow and 73 animals treated with heterologous bone marrow were examined microscopically. The observation time was 130 days.

RESULTS

Mortality

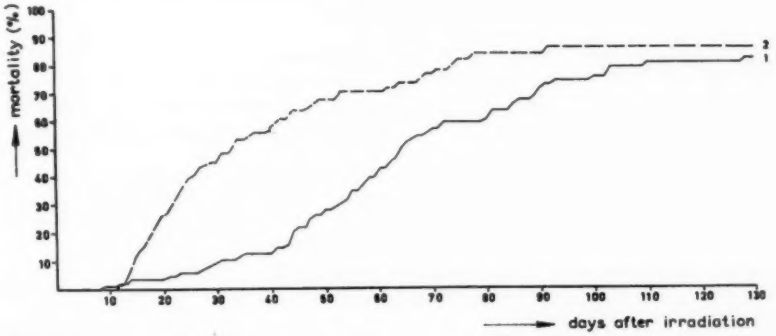
During the 130 days of the experiment no mortality occurred among the unirradiated, untreated controls and the mice treated with isologous bone marrow.

All irradiated, untreated control mice were dead on the 11th day after irradiation. Due to the rather small number in the latter group (25 mice), no attempt was made to express spontaneous mortality in a curve, by correcting for the animals that were killed. However, when the control animals of other experiments performed in the same year

are taken into consideration, it appears that, after total-body irradiation with 675 r, 100 percent mortality occurs before the 16th day.

Mortality curves of mice treated with homologous or heterologous bone marrow were constructed by computing the daily death rate after subtraction of the number of mice killed on the preceding day from the total number of mice alive.

After treatment with homologous bone marrow, 83 percent of the mice died spontaneously, between the 8th and 128th days (text-fig. 1). Since only 10 percent of the animals died before the 30th day, maximum mortality occurred after the 1st month.



TEXT-FIGURE 1.—Mortality of male CBA mice after total-body irradiation (675 r) and treatment with: (1) homologous (C57BL) bone marrow (22.5×10^6 cells); (2) heterologous (rat) bone marrow (50×10^6 cells).

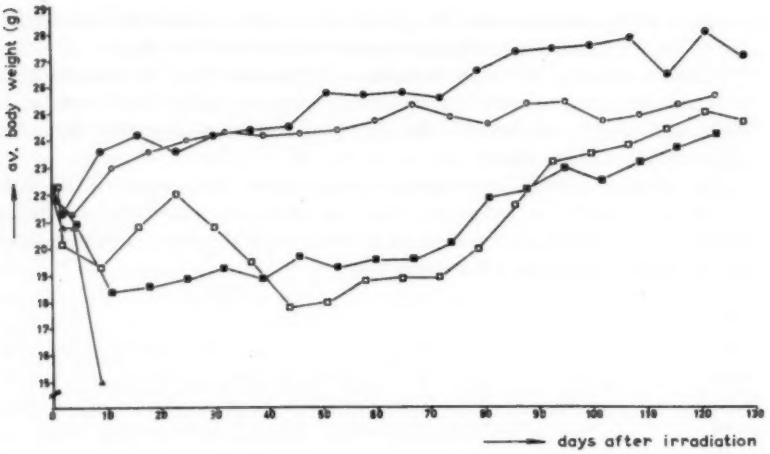
After treatment with heterologous bone marrow, 87 percent of the animals died, between the 11th and the 92d day (text-fig. 1). In this group maximum mortality occurred in the 1st month, since 46 percent of the mice died before the 30th day.

Body-Weight Changes

The weight curves of the mice in the various experimental groups are given in text-figure 2. The irradiated, untreated mice lost weight rapidly during the 1st week.

The weight curve of the mice treated with isologous bone marrow at first paralleled that of the unirradiated, untreated controls, but after the 70th day a slight difference was found. The mice treated with homologous bone marrow lost weight up to the 9th day; thereafter they gained weight until a second period of weight loss occurred after the 23d day. After the 72d day the body weight increased again, approaching normal values toward the end of the experiment.

The mice treated with heterologous bone marrow also showed a loss of body weight. Contrary to the animals of the former group, no temporary recovery of weight was noted in this group. The survivors gained weight after the 70th day.



TEXT-FIGURE 2.—Response of body weight in male CBA mice after total-body irradiation (675 r) and treatment with bone marrow. The points represent the average weight of animals alive on the specified day: ● = unirradiated, untreated; △ = irradiated, untreated; ○ = irradiated and treated with isologous bone marrow (6.4×10^6 cells; □ = irradiated and treated with homologous (C57BL) bone marrow (22.5×10^6 cells); ■ = irradiated and treated with heterologous (rat) bone marrow (50×10^6 cells).

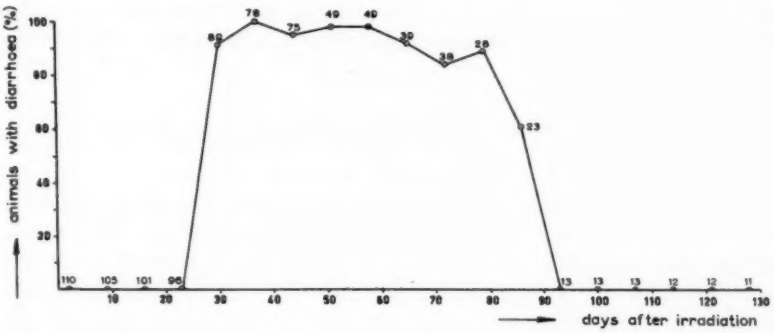
Symptoms of Secondary Disease

A large number of mice after treatment with both homologous or heterologous bone marrow appeared to be ill at the periods of high mortality and weight loss. The animals were emaciated, showed decreased activity, and most of the time were huddled up in their cages. In the 2d month skin changes appeared. The fur lost its gloss and became ruffled. Extensive depilation occurred in some animals. The depigmentation of the fur, which occurs after irradiation, was found much later in the mice treated with foreign bone marrow than in those treated with isologous bone marrow, indicating a disturbance of growth of the hair follicles. The skin showed fissures and was thinned and scaly.

The subcutaneous tissues were fibrotic. Some mice developed cutaneous ulcers which were covered with an inspissated exudate. In many mice the skin was infested with parasites (*Mycoptes musculinus*).

After the 3d week, most of the mice treated with homologous bone marrow excreted voluminous light-colored stools of soft consistency, denoted as diarrhea. The "diarrhea" coincided with the 2d period of weight loss and lasted until the 85th day (text-fig. 3).

In this particular experiment diarrhea was less frequent in the mice treated with heterologous bone marrow. Although between the 25th and 88th days a number of animals had diarrhea, the condition seemed to be less severe than after treatment with homologous bone marrow. In



TEXT-FIGURE 3.—Incidence of diarrhea in male CBA mice after total-body irradiation (675 r) and treatment with homologous (C57BL) bone marrow (22.5×10^6 cells). Diarrhea was considered present when feces adhered to the base of the tail. Numbers in graph indicate total number of mice inspected on specified day.

other experiments, not reported here, severe diarrhea also was observed in mice treated with rat bone marrow.

Hematological Data

Radiation-Induced Changes

In all groups the mononuclear cells had diminished, 24 hours after irradiation, to a small fraction of the normal value. After 24 hours the reticulocytes fell to half the normal value, or less, and at 48 hours few, if any reticulocytes, were observed. After the 3d day severe neutropenia developed in all animals. The 2 animals treated with isologous bone marrow and examined at 24 and 48 hours, respectively, showed a slight neutrophilia. The thrombocytes showed a gradual decline between the 3d and 9th days.

Recovery After Bone Marrow Treatment

The earliest indication of a proliferation of the bone marrow graft was the reappearance of reticulocytes, on the 5th day after irradiation, in animals of the treated groups. In all animals treated with isologous bone marrow and in a number treated with homologous and heterologous bone marrow, a reticulocytosis occurred during the 2d and 3d week. The number of neutrophil granulocytes increased after the 5th day, in all animals. Recovery of the thrombocyte values was initiated after the 9th day, in animals of all treatment groups.

In animals treated with isologous bone marrow, a slight increase in the number of mononuclear cells was observed at the end of the 2d week, with a slow return to normal values. In these animals the values of all cells had returned to normal by the end of the 4th week. The period of recovery varied for the different cell elements: mononuclear cells, 3 to 4

weeks; thrombocytes, 15 to 18 days; and neutrophil granulocytes, 7 to 9 days. The reticulocytosis disappeared after 3 to 4 weeks. Anemia was never apparent.

Abnormal Values After Treatment With Bone Marrow

(*a*) *Red blood cells*

A number of animals treated with homologous or heterologous bone marrow showed anemia after the 3d week after irradiation. Low hemoglobin values (not less than 10 gm. %) as well as low erythrocyte counts (not less than 4×10^6 cells) occurred (table 1). In 3 mice treated with homologous bone marrow, which were examined on days 12, 15, and 17, low hemoglobin values, but normal erythrocyte values, were noted. In 3 mice, examined on days 30, 31, and 50, that were treated with heterologous bone marrow, the anemia was very severe (less than 5 gm. percent hemoglobin and less than 2×10^6 erythrocytes).

TABLE 1.—Occurrence of anemia* in irradiated (675 r) CBA mice treated with bone marrow

	Number of animals with low Hb./total No.			Number of animals with erythropenia/total No.		
	Days after irradiation:			Days after irradiation:		
Bone marrow†	15–30	30–60	60–130	15–30	30–60	60–130
Isologous	0/5	0/10	0/8	0/5	0/10	0/8
Homologous	3/4	6/10	6/8	0/4	4/10	3/8
Heterologous	2/5	6/10	9/15	3/6	6/10	4/15

*Values less than 16 gm. percent hemoglobin and less than 7×10^6 erythrocytes per mm^3.
†Isologous bone marrow, 6.4×10^6 cells; homologous (C57BL) bone marrow, 22.5×10^6 cells; heterologous (rat) bone marrow, 50×10^6 cells.

In the 1st month after irradiation a number of mice treated with homologous or heterologous bone marrow showed reticulocytopenia; animals treated with isologous bone marrow had normal or high reticulocyte counts (table 2). Two mice of the latter group had slightly subnormal values, 84×10^3 cells at the 43d and 58th day, respectively.

TABLE 2.—Occurrence of abnormal* reticulocyte values in the peripheral blood of irradiated (675 r) CBA mice treated with bone marrow

	Number of animals with reticulocytopenia/total No.			Number of animals with reticulocytosis/total No.		
	Days after irradiation:			Days after irradiation:		
Bone marrow†	15–30	30–60	60–130	15–30	30–60	60–130
Isologous	0/5	2/10	0/8	4/5	0/10	0/8
Homologous	1/4	3/10	0/8	2/4	5/10	7/8
Heterologous	2/6	2/10	1/15	4/6	6/10	7/15

*Values below 9×10^4 or above 20×10^4 cells per mm^3.
†For experimental details *see* table 1.

However, much lower values were noted after treatment with foreign bone marrow—between 48×10^3 and 0 cells.

Contrary to the findings after treatment with isologous bone marrow, a large number of mice treated with homologous or heterologous bone marrow had a high number of reticulocytes after the 4th week (table 2).

The results indicate that after treatment with foreign bone marrow a few animals had an aplastic type of anemia. More frequently, however, the anemia seemed to be the result of increased destruction of red blood cells in animals which, in view of the high reticulocyte counts, apparently had actively proliferating red bone marrow.

(*b*) *Thrombocytes*

During the first 3 weeks after treatment with heterologous bone marrow, thrombocyte values were always lower than in animals treated with isologous bone marrow. After the 3d week after treatment with homologous and heterologous bone marrow, a number of the animals showed a thrombocytopenia (table 3). Extremely low values or a complete absence of platelets were found in 4 animals which received heterologous bone marrow on days 22, 23, 30, and 31.

TABLE 3.—Occurrence of abnormal* thrombocyte values in the peripheral blood of irradiated (675 r) CBA mice treated with bone marrow

	Number of animals with thrombocytopenia/total No.			Number of animals with thrombocytosis/total No.		
	Days after irradiation:			Days after irradiation:		
Bone marrow†	15–30	30–60	60–130	15–30	30–60	60–130
Isologous	2/5	0/9	0/8	1/5	1/9	0/8
Homologous	0/4	2/10	1/8	0/4	2/10	1/8
Heterologous	6/6	2/9	3/15	0/6	4/9	5/15

*Values below 8×10^5 or above 20×10^5 cells per mm^3.
†For experimental details *see* table 1.

Abnormally high numbers of thrombocytes were observed in a few animals treated with isologous or homologous bone marrow and, more frequently, in animals treated with heterologous bone marrow.

(*c*) *Neutrophil granulocytes*

After the 2d week neutropenia was observed, on days 15, 24, and 30, in 3 mice treated with heterologous bone marrow. After the 3d week mice of all treatment groups showed neutrophilia (table 4). Neutrophilia was more frequent and more severe after treatment with foreign bone marrow.

(*d*) *Mononuclear cells*

Since it is impractical to differentiate reliably between monocytes and lymphocytes in mice, these cells were counted together as mononuclear cells. Recovery of the mononuclear cells was greatly retarded in animals

TABLE 4.—Occurrence of abnormal* values of neutrophils in the peripheral blood of irradiated (675 r) CBA mice treated with bone marrow

	Number of animals with neutropenia/total No.			Number of animals with neutrocytosis/total No.		
	Days after irradiation:			Days after irradiation:		
Bone marrow†	15–30	30–60	60–130	15–30	30–60	60–130
Isologous	0/5	0/10	0/8	1/5	2/10	2/8
Homologous	0/4	0/10	0/8	2/4	6/10	6/8
Heterologous	2/5	1/10	0/15	1/5	6/10	8/15

*Values below 2×10^3 or above 8×10^3 cells per mm^3.
† For experimental details *see* table 1.

treated with foreign bone marrow compared to those treated with isologous bone marrow (table 5). Until the end of the experiment, the majority of the mice in the former group showed abnormally low counts of mononuclear cells. In general the values remained much lower after treatment with heterologous than after treatment with homologous bone marrow.

TABLE 5.—Occurrence of low* values of mononuclear cells in the peripheral blood of irradiated (675 r) CBA mice treated with bone marrow

	Number of animals with monocytopenia/total No.		
	Days after irradiation:		
Bone marrow†	15–30	30–60	60–130
Isologous	4/5	1/10	0/8
Homologous	3/4	8/10	4/8
Heterologous	5/5	9/10	7/15

*Values below 8×10^3 cells per mm^3.
†For experimental details *see* table 1.

(*e*) *Eosinophil granulocytes*

After the 9th day, 1 of 23 animals treated with homologous bone marrow and 4 of 24 animals treated with heterologous bone marrow showed abnormally large numbers of eosinophils (more than 10% of the total number of white blood cells).

Typing of Mouse and Rat Granulocytes and Erythrocytes

After treatment with rat bone marrow, a small percentage of alkaline-phosphatase-positive granulocytes was found as early as 48 hours after irradiation. Between the 2d and 7th days the determination was impossible in view of the small number of circulating white cells. After the 7th day and until the end of the experiment, the neutrophils were tested for alkaline phosphatase in 27 mice. In 5 mice, tested on days 24, 57, 86, 106, and 129, no positive cells were observed.

Rat erythrocytes could be demonstrated, from the 14th day; they were

typed serologically in 16 mice, between the 14th and 129th days. In 7 mice, tested on days 23, 37, 51, 58, 105, 106, and 128, only rat erythrocytes could be demonstrated and in 7 mice, tested on days 30, 31, 38, 85, 86, 129, and 129, only mouse erythrocytes. Two mice, tested on days 14 and 72, appeared to have a mixed population of rat and mouse erythrocytes.

Unfortunately the data are insufficient to justify any inference concerning the relation between the type of functioning hematopoietic tissue and the hematological abnormalities. However, they indicate that in this experiment a reversal from rat-to-mouse type hematopoiesis has occurred frequently.

Pathological Data

Septicemia

It has been shown that after total-body irradiation, 675 r, 80 percent of CBA mice die of bacteremia, with group G streptococci (*28*). In view of *post-mortem* changes, bacteriological examination of blood samples could not be carried out regularly in our experiments. Septicemia was therefore also presumed to exist when pathological changes typical for this condition were noted. In the irradiated, untreated control mice signs of bacteremia were observed from the 8th day. Gross examinations showed that 8 of 11 animals, autopsied between the 8th and 11th days, had multiple grayish-white foci in the liver, the kidneys, the intestines, and less frequently in other organs.

Microscopically these lesions consisted of necrotic foci in which a large number of bacteria had accumulated. Characteristically cellular exudation was absent. In the liver, necrotic changes comprised both the liver cells and the connective-tissue stroma (fig. 1). In addition clumps of bacteria were seen in blood vessels of most organs.

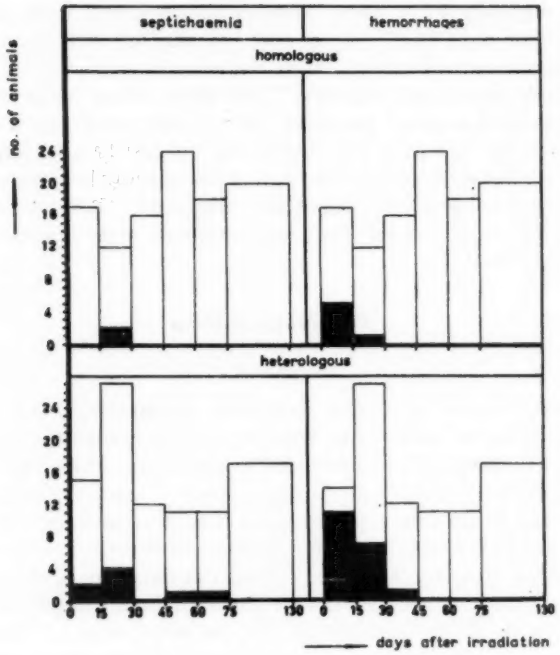
None of the animals treated with isologous bone marrow showed evidence of septicemia. In contrast, after treatment with foreign bone marrow septicemia could be demonstrated in a number of mice.

In 2 mice treated with homologous bone marrow (21st and 23d day) and in 8 mice treated with heterologous bone marrow (on days 15, 16, 16, 21, 26, 29, 55, and 74) septicemia was apparent. The necrotic lesions in the mice that died on the 29th and 74th day revealed slight polynuclear infiltration. In the mouse that died on the 74th day a bacterial endocarditis was found.

The data show that the incidence of septicemia was much greater in mice treated with heterologous than in those treated with homologous bone marrow. Furthermore, in both groups of animals generalized infection occurred chiefly before the 30th day after irradiation (text-fig. 4).

Hemorrhagic Diathesis

In untreated mice and in a number of mice treated with foreign bone marrow, a hemorrhagic disorder was revealed by prolonged bleeding from



TEXT-FIGURE 4.—Incidence of septicemia and hemorrhagic diathesis in male CBA mice after total-body irradiation (675 r) and treatment with homologous (C57BL, 22.5×10^6 cells) or heterologous (rat, 50×10^6 cells) bone marrow. *White* areas indicate negative findings and *black*, positive findings in animals examined during indicated interval.

the tail, after a blood sample was taken, and by grossly or microscopically visible multiple hemorrhages in the skin, the wall of the stomach, the intestines, the testes, and many other organs. The bleeding tendency was always accompanied by a peculiar lesion in the stomach. Widespread cystic dilatation of the gastric glands occurred, the spaces of which were lined by flattened epithelium and which often contained masses of erythrocytes. Probably the lesion was caused by superficial hemorrhages of the mucosa, giving rise to obstruction of the neck of the glands.

In the irradiated, untreated controls hemorrhagic diathesis was first seen on the 7th day and occurred in 12 of 14 animals examined between the 7th and 11th days.

With the possible exception of 1 animal, which showed a few dilated gastric glands, a bleeding tendency could never be detected after treatment with isologous bone marrow.

After treatment with homologous bone marrow, a hemorrhagic tendency was revealed in 6 animals (on days 7, 9, 9, 12, 12, and 23), and after

treatment with heterologous bone marrow, in 19 animals (on days 5, 7, 9, 9, 12, 14, 15, 15, 15, 15, 15, 16, 16, 19, 21, 21, 23, 30, and 33).

As with septicemia, hemorrhagic diathesis occurred in the 1st month after treatment with foreign bone marrow and prevailed in those mice that received heterologous bone marrow (text-fig. 4). Both disorders were invariably associated with bone marrow aplasia, as will be shown in the next section.

Bone Marrow

Mice of all groups showed greatly decreased cellularity of the bone marrow 24 hours after irradiation. Only mature granulocytes and megakaryocytes remained. The next day the marrow space was occupied by dilated capillaries between which reticular cells and a few megakaryocytes were dispersed. The megakaryocytes showed pyknosis and other nuclear alterations.

In untreated mice an aplastic bone marrow persisted until the death of the animals. A bone marrow smear of 1 animal, which was killed on the 10th day, revealed a few groups of intact erythroblasts.

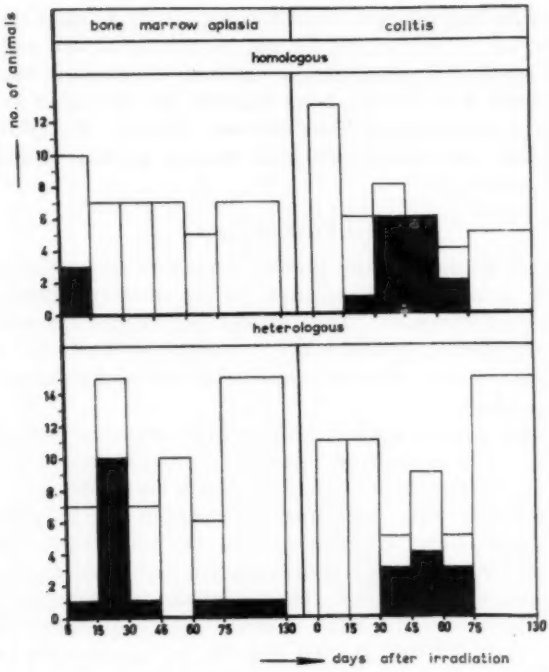
After treatment with isologous, homologous, or heterologous bone marrow, recovery was initiated on the 4th day, when collections of swollen reticular cells and undifferentiated stem cells (hemocytoblasts) appeared in the marrow tissue. On the 5th day the bone marrow showed normal or increased cellularity. Predominantly immature forms of all series could be found (fig. 2). On the 7th day maturation had occurred and, except for a notable increase of cellularity, the tissue had an almost normal appearance.

In 3 animals treated with homologous bone marrow (on days 7, 9, and 12) and in 15 animals treated with heterologous bone marrow (on days 9, 16, 16, 21, 22, 23, 24, 25, 29, 29, 30, 44, 44, 74, and 92) a severely decreased cellularity or a total absence of hematopoietic tissue in the bone marrow was observed after the 5th day (fig. 3). In one of the mice which showed bone marrow aplasia on the 44th day, an extensive extramedullary hematopoiesis was noted. The data of this mouse were therefore not included in text-figure 5.

Besides acellularity of the bone marrow, several mice showed absence of mature elements, chiefly of the granulopoietic series and an increase of immature precursors: 1 mouse on the 28th day and 5 mice on days 38, 44, 47, 53, and 53 after treatment with homologous or heterologous bone marrow, respectively.

Extensive necrosis of either the sternal or femoral marrow, or both, was noted in 4 mice treated with homologous bone marrow (on days 44, 72, 79, and 79). In 3 mice, marrow of normal appearance was seen in other bones. In 3 mice treated with homologous bone marrow (on days 72, 92, and 92) a patchy replacement of bone marrow by fibroblasts and a hyaline intercellular substance was noted. Possibly this was the result of a preceding necrosis.

Text-figure 5 shows that bone marrow aplasia, after treatment with



TEXT-FIGURE 5.—Incidence of bone marrow aplasia and chronic colitis in male CBA mice after total-body irradiation (675 r) and treatment with homologous (C57BL, 22.5×10^6 cells) or heterologous (rat, 50×10^6 cells) bone marrow. *White* areas indicate negative findings and *black*, positive findings in animals examined during indicated interval.

foreign bone marrow, occurred before the 30th day after irradiation, in most of the mice. Most animals examined thereafter showed a hypercellular bone marrow. The hypercellularity was generally due to an increase in the white blood cell series, but in a few instances it was due to erythroblastosis.

Except for the spleen, which will be described in the next paragraph, *extramedullary hematopoiesis* was frequently observed in the liver, thymus, lymph nodes, submucosa of the large intestine, and between the muscle fibers of the chest in the vicinity of the sternum, in mice treated with homologous or heterologous bone marrow. In the liver small periportal collections of reticular cells, erythroblasts, myelocytes, and granylocytes were regularly seen after the 2d week. In mice treated with homologous bone marrow extensive hematopoiesis was often seen in the liver.

Spleen

Although slight hematopoietic activity in normal mice may usually be noticed in the splenic red pulp throughout their lifespan, during the

1st month after irradiation and bone marrow transplantation, the spleen appears to be a blood-forming organ of prime importance. The first signs of myelopoietic activity were discernible on the 4th day and the recovery pattern was the same as that described for bone marrow. In the following days the red pulp became crowded with hematopoietic cells. In general erythroblasts were much better represented than in the bone marrow.

In animals treated with isologous bone marrow, myelopoietic activity in the spleen diminished to normal proportions toward the 6th week. In contrast extensive myelopoiesis persisted till the end of the experiment in most animals that were treated with homologous or heterologous bone marrow (fig. 4). In a number of animals the blood-forming tissue was not evenly distributed, but occurred in focal cell collections. The intervening red pulp sometimes was hyalinized. In those animals which showed bone marrow aplasia, myelopoietic activity was usually absent or scanty in the spleen; fibrosis of the red pulp had occurred in a number of mice.

Necrosis of hematopoietic tissue in the red pulp was seen in 2 mice that received homologous bone marrow (on days 7 and 9). In 1 animal treated with homologous bone marrow and examined on the 44th day, an eosinophilic substance resembling fibrin had been deposited around central arteries in edematous stroma (fig. 4). These foci were surrounded by dense collections of myeloid cells.

From the observation of the spleens of many animals, treated with foreign bone marrow, the impression was gained that abnormal quantities of hemosiderin had been deposited in endothelial cells of the red pulp. The large variation in spleen volume prevented a reliable judgment of the extent of the siderosis. The white pulp will be considered in another paragraph.

Thymus

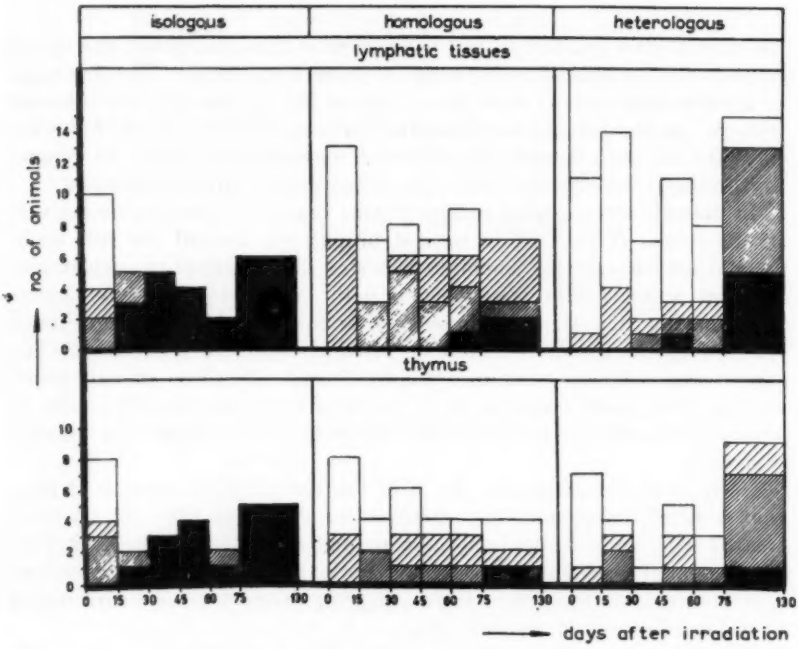
At 24 hours after irradiation, a small thymus, which showed almost complete disappearance of thymocytes from the cortex, was seen in all animals.

In animals treated with isologous bone marrow, cortical regeneration started on the 7th day, differentiation in cortex and medulla had occurred on the 12th day, and complete restoration of size and structure on the 24th day.

After treatment with foreign bone marrow complete regeneration of the thymus was observed in only 2 animals (1 of which received homologous and 1 heterologous bone marrow) on the 100th day. In most animals either total atrophy of the thymic cortex persisted or only slight or incomplete regeneration occurred (text-fig. 6).

Lymph Nodes and Lymphatic Tissue of Spleen and Intestinal Tract

The number of mature lymphocytes in the lymphatic tissues was greatly reduced 24 hours after irradiation. Reticular cells, varying num-



TEXT-FIGURE 6.—Recovery of lymphatic tissues and thymus in male CBA mice after total-body irradiation (675 r) and treatment with isologous (6.4×10^6 cells), homologous (C57BL, 22.5×10^6 cells), or heterologous (rat, 50×10^6 cells) bone marrow: □ = no regeneration: atrophic reticular tissue, no lymphopoietic activity; ▨ = scanty regeneration: tissues generally atrophic, some mitotic activity, a few lymphoblasts and small focal collections of lymphocytes; ▣ = advanced regeneration: dense collections of lymphoid cells, germinal centers; ■ = complete regeneration: structure similar to that of unirradiated controls. Each bar represents findings in animals investigated during indicated interval.

bers of pyknotic lymphocytes, and a few macrophages with phagocytized nuclear remnants remained. Usually the capillaries were dilated and slight infiltration with polynuclear cells was seen.

The lymphatic tissues remained atrophic in the irradiated, untreated mice. However, 1 of the animals showed diffuse collections of lymphocytes and some mitotic activity in the cortex of a lymph node, on the 11th day.

After treatment with isologous bone marrow, recovery of the lymphatic tissues started on the 7th day—shown by the appearance of small groups of lymphoblasts and mitotic activity. Advanced regeneration had occurred on the 12th day, when dense collections of mature lymphocytes were seen, and recovery was completed in all mice after the 23d day (text-fig. 6).

Regeneration of the lymphatic tissues was retarded or failed to occur

in mice treated with foreign bone marrow (text-fig. 6). In a number of animals examined after the 1st month, the lymphatic tissues consisted of a reticular stroma, devoid of lymphoid cells, which was sometimes infiltrated with polynuclear cells (fig. 4). In other animals extensive fibrosis of lymphatic tissue was seen. A few animals that were treated with homologous bone marrow showed one or more focal collections of lymphoid cells in the cortex of a lymph node, which otherwise was completely atrophic.

Often the atrophic tissues showed dilatation or increase of small blood vessels. Sometimes perivascular infiltration with plasma cells was observed, which in many cases was accompanied by the occurrence of large numbers of swollen reticular cells and macrophages. These have been described by Congdon, Makinodan, and Gengozian (*29*) as epithelioid cells (fig. 5).

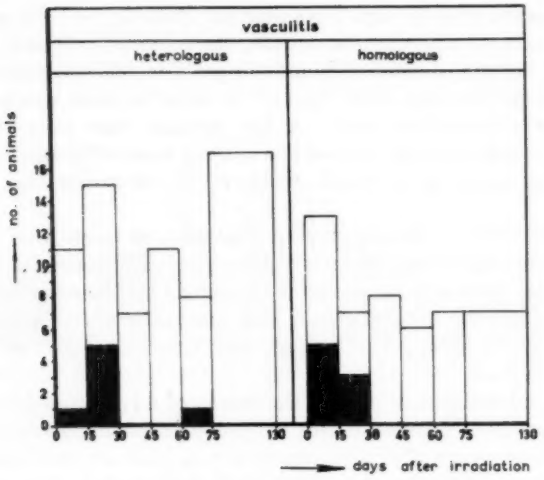
In 1 animal necrosis of part of the cortex of a lymph node was seen on the 28th day after treatment with heterologous bone marrow.

Complete recovery of the lymphatic tissues had occurred after the 70th day in a few animals treated with homologous bone marrow. Although in 1 animal the lymphatic tissues were restored on the 60th day, complete recovery in mice treated with heterologous bone marrow usually did not occur until after the 100th day. Text-figure 6 illustrates clearly that regeneration of the lymphatic tissues occurred earlier after treatment with homologous bone marrow than after treatment with heterologous bone marrow.

Blood Vessels

A peculiar lesion of the blood vessels was seen in a number of animals treated with foreign bone marrow. Usually arterioles and capillaries but, in a few mice, also larger arteries, in the submucosa of the stomach, intestines, liver, lymph nodes, testis, myocardium, lung, and subcutaneous tissue were affected. The lesion was characterized by necrosis of the vascular wall, which showed nuclear fragmentation and increased staining with the PAS-reaction, and infiltration of the vascular and perivascular tissues with polynuclear leukocytes or plasma cells (fig. 6). In some mice the endothelium was swollen; in others, obliteration occurred as the result of intimal proliferation or thrombosis. This necrotizing vasculitis was observed as frequently after treatment with homologous bone marrow (8 mice on days 7, 7, 9, 9, 12, 17, 23, and 30) as after treatment with heterologous bone marrow (7 mice on days 15, 16, 17, 18, 22, 29, and 74) and occurred mainly before the 30th day (text-fig. 7).

In 4 of these animals bacteriological examination of the heart blood was performed; 1 showed a *Streptococcus viridans* septicemia. The finding of clumps of bacteria in the wall and the lumen of a necrotic artery in this animal may be significant. Association of vascular alterations with septicemia was apparent in another animal, which suffered from bacterial endocarditis. In 4 of 14 mice, which showed vascular lesions, bone marrow aplasia was present.



TEXT-FIGURE 7.—Incidence of vascular lesions in male CBA mice after total-body irradiation (675 r) and treatment with homologous (C57BL, 22.5 × 10^6 cells) and heterologous (rat, 50 × 10^6 cells) bone marrow. *White* areas indicate negative findings and *black*, positive findings in animals examined during indicated interval.

Intestines

In all irradiated animals a few crypts of the small intestine showed slight nuclear pyknosis or fragmentation and epithelial desquamation at 24 and 48 hours. Loss of a few crypts also was apparent in the large intestine, but occurred later, between the 4th and 9th days. Slight infiltration of degenerated crypts with polynuclear leukocytes was seen in 1 untreated mouse (2d day), 1 mouse treated with isologous bone marrow (15th day), and 2 mice treated with homologous bone marrow (7th and 9th day). After the 15th day the intestines were normal in mice treated with isologous bone marrow.

Concurrently with the appearance of diarrhea, alterations of the large intestine and the terminal ileum were observed in animals treated with homologous or heterologous bone marrow. On gross examination few changes were visible. A number of animals showed congestion of the cecum and ascending colon. Sometimes extreme dilatation of the small intestine due to accumulation of gas and intestinal juice was seen. Gross changes of the mucous membrane were never seen. Microscopically the mucous membrane appeared to be patchy. The lesion was characterized by diffuse infiltration of the mucosa and submucosa, with varying amounts of neutrophil and sometimes eosinophil granulocytes, plasma cells, and mononuclear cells (fig. 7).

Degeneration of crypt epithelium with accumulation of pyknotic and desquamated epithelial cells in the lumen of the glands was often seen, and resulted in the loss of an appreciable number of crypts (fig. 8). In

other places affected crypts were covered with aspecific low cuboidal or squamous epithelial cells. In addition a cystic change of atrophic crypts has been observed (fig. 8). Dense infiltration of the wall and the space of crypts with polynuclear leukocytes frequently resulted in a resemblance to the crypt abscesses seen in ulcerative colitis. The lamina propria often showed an increase of connective-tissue stroma. This undoubtedly was partly due to loss of crypts. However, dense collections of fibroblasts suggested that active fibrosis was also present.

Edema of the submucosa and dilatation of lymphatic vessels was often noted (fig. 7). The intestinal contents frequently showed a considerable admixture with polynuclear leukocytes.

The severity of the lesion varied from a slight cellular infiltration to extensive destruction of glandular tissue. This apparently could result in the widespread mucosal atrophy, which was observed in a few animals after the 2d month.

Colitis was first seen on the 27th day after treatment with homologous and on the 44th day after treatment with heterologous bone marrow. After the 70th day no active colitis was noted (text-fig. 5), though a few mice showed mucosal atrophy. Comparison of text-figures 3 and 5 demonstrates that the periods of diarrhea and colitis roughly coincide. Colitis occurred in 14 of 18 mice treated with homologous bone marrow and examined between the 30th and 75th days, as against 10 of 19 mice treated with heterologous bone marrow which were examined in the same period (text-fig. 5). The intestinal lesions were more extensive in the former group. This agrees with the higher incidence of severe diarrhea in these mice.

After treatment with heterologous bone marrow, extensive telangiectasis of blood vessels in the wall of the small and large intestine was observed in 5 animals (on days 70, 99, 103, 103, and 118). The vascular dilatations were located in the mucosa and submucosa. Apparently development of these vascular scars was in progress in the animal examined on the 70th day; the mucosa of the cecum showed a low-grade colitis and atrophic areas. Several dilated vessels contained partly canalized thrombi and infiltration with polynuclear leukocytes. Whether the telangiectases are the result of chronic colitis or of necrotizing vasculitis is not clear at present.

Liver

The occurrence of focal necrosis of the liver during the 1st month, in mice with septicemia, has been described in a preceding paragraph. The focal lesions showed loss of both liver cells and supporting tissue and contained clumps of bacteria. In addition, 2 untreated mice and 1 mouse treated with homologous bone marrow showed hepatic necrosis without evidence of bacterial growth (on days 10, 11, and 17). Similarly in these 3 animals the necrotic changes comprised both parenchyma and stroma. Furthermore, 2 of the 3 mice showed histological evidence of septicemia; necrotic foci which contained clumps of bacteria were seen outside the

liver in 1 animal, and vascular necrosis had occurred in the other. As will be discussed later, these vascular lesions probably are related to septicemia.

A different lesion was observed during the 2d and 3d month, in animals treated with foreign bone marrow. On gross examination several sharply defined, grayish foci were seen in a number of mice. In other animals the lesions were more extensive. The liver had a mottled aspect caused by depressed, red, and slightly elevated, yellow-to-gray areas. Microscopically the lesions showed varying extents of lobular disarray and necrosis of liver cells, with preservation of the condensed supporting tissue (fig. 9). Sometimes an increase of structures resembling bile ducts was seen. In cases of extensive necrosis the center of the lobules contained severely dilated sinusoids (fig. 9). In 1 animal early cirrhosis was evidenced by proliferation of fibroblasts.

The late type of hepatic necrosis, which resembled acute yellow atrophy, in several respects, was seen in 3 mice treated with homologous (on days 51, 64, and 65) and in 4 mice treated with heterologous bone marrow (on days 38, 44, 47, and 52).

Lungs

Bronchopneumonia frequently leading to lung abscesses was noted between the 25th and the 81st days in 2 mice treated with heterologous and in 29 mice treated with homologous bone marrow. It should be stressed in this respect that the experiments with homologous and heterologous bone marrow were not carried out simultaneously. There is no reason to assume that the different incidence of infections of the lung is related to the different origin of the bone marrow. In these experiments neither the mice treated with isologous bone marrow nor the unirradiated controls developed pneumonia.

From tissue of pneumonic lungs a gram-negative bacillus was isolated repeatedly. This organism is probably identical with a commensal microorganism that is normally present in nose and pharynx of our CBA mice.

Pulmonary edema was the cause of death in 7 mice treated with foreign bone marrow. This was probably due to acute myocardial failure, which will be discussed in the next paragraph.

Heart

Focal degeneration with subsequent calcification of muscle fibers of the myocardium occurs normally in young CBA mice. Extensive calcification may be seen in mice over 1 year old.

The incidence of focal myocardial calcification was not significantly increased in mice treated with isologous bone marrow. In contrast, after the 14th day, extensive calcification accompanied by myocardial fibrosis was observed in the majority of mice treated with foreign bone marrow. This may have resulted in acute myocardial failure, as evidenced by pulmonary edema, in 4 mice treated with homologous (on days 64, 69, 70, and 110) and 3 mice treated with heterologous bone marrow (on days

29, 53, and 71). Another mouse treated with heterologous bone marrow showed, apart from severe myocardial calcification, interstitial fibrosis of the lung, indicating long-continued myocardial insufficiency.

Testis

The radiation-induced changes of the testis were initially identical in all experimental groups. The number of spermatogonia was distinctly reduced at 48 hours; 5 days after irradiation all spermatogonia had disappeared. On the 9th day a reduction in the number of primary spermatocytes was apparent. On the 12th day primary spermatocytes were absent and depletion of secondary spermatocytes, spermatids, and spermia was noted. Toward the 17th day spermatogenesis was totally absent and the seminiferous tubules were lined by sustentacular cells only.

In animals treated with isologous bone marrow, recovery of spermatogenesis first became apparent on the 20th day when small groups of spermatogonia were seen in a few tubules. Primary spermatocytes reappeared on the 24th, and secondary spermatocytes, spermatids, and spermia on the 37th day. For a long period spermatogenesis remained irregularly distributed. Toward the 70th day recovery was nearly complete, though a few empty tubules per section were regularly noted until the end of the experiment.

In animals treated with homologous or heterologous bone marrow, recovery of spermatogenesis also occurred, but was generally delayed. The extent of the recovery, however, was much more variable compared to mice treated with isologous bone marrow.

Miscellaneous Observations

Besides small hemorrhages in mice that had hemorrhagic diathesis, the adrenals never showed obvious changes.

In one of the animals treated with homologous bone marrow a purulent epididymitis was observed (51st day).

A submandibular abscess was seen in a mouse, autopsied on the 52d day, that had been treated with homologous bone marrow. The abscess probably had its origin in the submandibular gland.

DISCUSSION

Under the conditions of our experiments, total-body irradiation with 675 r caused the death of all mice within 15 days. The animals died of the bone marrow syndrome, which is characterized by septicemia, hemorrhagic diathesis, and severe anemia.

Treatment with isologous bone marrow prevents the bone marrow syndrome by restoring the hematopoietic system of the irradiated mice before the detrimental effects of bone marrow aplasia supervene. The hematological data show that after the 4th week recovery of the hemato-

poietic system is quantitatively complete. Except for the changes in the testis and the depigmentation of the fur, no pathological changes could be demonstrated after the 4th week. The animals did show, however, a lag in the weight curve compared to that of the unirradiated controls. This is probably related to those late irradiation effects, which are not prevented by bone marrow transplantation.

In contrast, mice treated with homologous or heterologous bone marrow became ill and showed a high mortality after the initial recovery. The results of this investigation clearly indicate that mice treated with foreign bone marrow may have at least two different disease entities. The mortality which occurred during the 2d half of the 1st month was associated with a bone marrow aplasia, which closely resembled the bone bone marrow syndrome after lethal doses of total-body irradiation. The mortality during the 2d and 3d month was in general associated with a second more complex syndrome, which was characterized by diarrhea, colitis, hepatic necrosis, dermatitis, and pneumonia. Apparently, in mice treated with heterologous bone marrow the earlier occurring illness was predominant, and the second syndrome occurred in those treated with homologous bone marrow (text-fig. 5). This explains the differences in the mortality pattern between the 2 groups (text-fig. 1): After treatment with heterologous bone marrow, maximum mortality occurred before the 30th day, while it occurred after the 30th day after treatment with homologous bone marrow. In addition, the weight curves of the animals of the 2 groups indicate both a complex disease pattern and a difference between the effects of homologous and heterologous bone marrow transplantation (text-fig. 2). It has to be stressed that the differences between mice treated with heterologous and homologous bone marrow are not a constant finding in other experiments in which the same radiation dose was employed. In addition, the early mortality has not been observed after irradiation with a dose of 800 r followed by treatment with rat bone marrow (*30*).

The delayed bone marrow failure.—In the 1st month after treatment with foreign bone marrow a number of mice showed aplastic anemia, neutropenia, and thrombocytopenia. This was associated with bone marrow aplasia or maturation arrest of the bone marrow, and pathological changes due to septicemia and hemorrhagic diathesis could be demonstrated. Since mortality and symptoms of bone marrow aplasia were noted after the 15th day, when all irradiated, untreated animals had died, it must be presumed that in these animals a functional graft of hematopoietic tissue has been temporarily present. Secondary rejection subsequent to initial proliferation of the bone marrow graft has been shown to occur by Congdon, Makinodan, and Gengozian (*29*).

The deleterious effect of the administration of foreign bone marrow after irradiation with median-lethal doses (*13*, *29*, *31*) may be similarly explained by the assumption that the immunological defense system of the host is not sufficiently eliminated by the irradiation. Its recovery is presumedly followed by an immunological reaction against the graft

resulting in its rejection (*32*). Even after a dose of 675 r many rat-mouse chimeras regained their own hematopoietic system, which indicates a gradual recovery of the immunological defense system of the host (*33*), and the potential ability of the host to reject the graft. In the present experiment this also occurred.

It is therefore likely that the mortality in the 1st month after treatment with homologous or heterologous bone marrow is either due to the failure of the graft to take or to its secondary rejection. The prolonged course of the delayed bone marrow syndrome, in animals treated with 675 r as compared to those subjected to a median-lethal radiation dose, may be explained by the more extensive destruction of the lymphatic tissues, which occurs after the higher dose (*29*). The animals die, apparently, because in the 1st month the host is not able to replace the rejected graft by its own hematopoietic tissue, in time. After the 1st month a low-grade immunological reaction of the host against the graft may result either in an anemia of a hemolytic type with a persisting graft or in a gradual destruction of the donor bone marrow with a concomittant replacement by hematopoietic tissue of the host (total or partial reversal) which could be shown to occur in mice treated with rat bone marrow. The anemia with high reticulocyte values, which was noted in a large number of mice treated with foreign bone marrow, after the 3d week, may be explained on this basis.

Treatment with heterologous bone marrow resulted in a much higher incidence of the delayed bone marrow syndrome than treatment with homologous bone marrow. Recently Welling *et al.* (*33*) found that reversion from donor-to-host type of hemoglobin occurs far less frequently in our homologous chimeras than in mice treated with rat bone marrow. In their experiments doses of X rays were used that were similar to those described in this paper. Possibly the immunological defense system remains less depressed after treatment with rat bone marrow. Rat cells may be antigenically much more effective than cells of the C57BL mouse (*2*). In the latter, rat bone marrow would elicit a stronger response of the immunological defense system of the host which, as has been pointed out earlier, is not entirely eliminated by the irradiation.

A reaction of the host against the graft has been postulated by a number of workers to account for the late secondary mortality after treatment with foreign bone marrow (*2*, *3*, *9*, *29*). Our experiments have shown, however, that bone marrow aplasia is mainly connected with mortality during the 1st month and that the mortality which occurs after the 30th day is the result of a completely different pathological process. The term secondary disease will be used only in connection with this latter syndrome.

The secondary disease.—The mortality of radiation chimeras after the 1st month after the irradiation is accompanied by multiple localized infections. In the material presented here pneumonia accounted for the majority of the deaths after treatment with homologous bone marrow. Chronic colitis which caused a prolonged period of diarrhea explains the

severe weight loss and emaciation of the animals. The inanition, apart from being the next important cause of death, possibly provoked or promoted the development of degenerative changes in the liver and the heart muscle. Hepatic necrosis and cardiac failure also may be held directly responsible for a number of deaths.

Skin transplantation experiments with radiation chimeras (*17, 34, 35*) have clearly shown that the take of a skin graft after the 1st month after the irradiation is determined primarily by the genotype of the donor bone marrow. This suggests that after the 1st month the host is immunologically much less active than the bone marrow graft.

Several workers have indicated that an immunological reaction of the graft against the host could be the cause of secondary mortality in irradiated mice treated with foreign bone marrow (*12, 13, 17, 19, 36*). Work on the effect of homologous lymph-node cells (*32*) and parabiosis (*30*) has provided further evidence that an immunological reaction of the graft against the host may provoke mortality and comparable symptoms, which occur in mice with secondary disease.

Immunological activity of injected foreign lymphoid cells against a tumor of the host's genotype is suggested in experiments with a transplantable lymphosarcoma of the C57BL mouse (*37*). Recently, antibody production by a graft of foreign spleen against tissues of the host has been demonstrated by Feldman (*38*).

The evidence presented points to a close relation between secondary *mortality* in the 2d and 3d month and an immunological reaction of the foreign graft against the host. It will be discussed whether such a relation also is apparent for the *symptoms* and the *pathological changes* of mice with the delayed foreign bone marrow reaction.

After the 1st month most of the animals showed a normal or an increased cellularity of *the bone marrow*. The frequent occurrence of prolonged bone marrow hyperplasia and excessive extramedullary hematopoiesis may be explained either by increased destruction of blood cells or by the high incidence of pneumonia, colitis, and other local inflammatory processes. The same explanation may be given for the high peripheral values of neutrophil granulocytes which occurred after the 30th day in mice treated with foreign bone marrow.

After the 3d week a large number of mice treated with foreign bone marrow developed an anemia with high reticulocyte values, the cause of which is still obscure. This type of anemia, contrary to the aplastic anemia in mice with bone marrow aplasia, never was of sufficient severity to account for the mortality in the 2d and 3d month.

The focal necrosis or fibrosis of bone marrow seen, in the 2d and 3d month, in a few mice treated with homologous bone marrow cannot be readily understood because mortality of bone marrow aplasia did not occur after the 1st month in these mice. The bone marrow necrosis might be the result of destruction of regeneratory foci of hematopoietic cells of the host. This could be a direct consequence of the immunological activity of the graft.

The recovery of the *lymphatic tissues* was much delayed or remained incomplete in animals treated with foreign bone marrow. Before the 60th day, only minimal proliferation of lymphoid cells could be seen, and the lymphatic tissues were atrophic and often showed fibrotic changes. After the 60th day complete recovery could be demonstrated only in a few mice. Incomplete regeneration was reflected in the prolonged low values of mononuclear cells in the peripheral blood.

Because of the importance of lymphoid cells in the production of transplantation immunity, these findings are particularly significant. It has been shown that the administration of homologous lymphoid cells to irradiated mice may result in an acute mortality (*32*, *39*). Since several workers have provided evidence to show that the lymphoid tissues are repopulated by donor cells after bone marrow transplantation (*5*, *40*, *41*), the latter procedure similarly can be expected to induce mortality. Secondary mortality in mice treated with foreign bone marrow appears, however, between the 30th and 100th day post irradiation, which is much later than in mice treated with sufficiently large numbers of homologous lymphoid cells. The retarded recovery of the lymphatic tissues in our chimeras, which were treated with bone marrow only, may explain, therefore, the chronic character and the prolonged course of the illness which occurred in the 2d and 3d month.

In mice treated with homologous bone marrow, the recovery of the lymphatic tissues in general occurred at an earlier date and usually was more extensive than in mice treated with heterologous bone marrow. Presuming that the lymphoid cells which proliferate after the 1st month are mainly of donor origin, the more severe character of secondary disease in mice treated with homologous bone marrow may be readily explained.

Particularly interesting in this connection are the collections of plasma cells and macrophages and the occasional deposition of fibrinoid substance in the spleen and lymph nodes, which also have been reported by Congdon and Urso (*22*). The authors relate their findings to an immunological "hypersensitive" state, and suppose that the pathological changes are associated with a chronic tissue antigen-antibody reaction *in vivo* leading to atrophy of the antibody-producing tissues.

The incidence of pneumonia after treatment with foreign bone marrow varies much in different experiments. In the experiments referred to in this paper, a high incidence was found in the 2d and 3d month, especially in mice treated with homologous bone marrow. Since pneumonia occurred neither in the unirradiated, untreated controls nor in the animals treated with isologous bone marrow, a decreased resistance to microorganisms which probably are commensals must be accepted. This agrees with the atrophy of the lymphatic tissues, which could result in the total absence of immunological defense. In addition, the foreign lymphoid cells which repopulate the lymphatic tissues might not be able to protect the host against his commensal microorganisms.

The diarrhea which occurred in the 2d and 3d month is clearly related

to the pathological changes of the *large intestine*, which were found concurrently. No straightforward explanation can be given for the inflammatory changes, the necrosis, and the atrophy of the mucous membrane. Possibly the intestinal epithelium is as sensitive as that of the skin to the immunological activity of a foreign bone marrow graft. In this case, the graft anti-host reaction would be directly responsible for the necrotic changes of the crypt epithelium, and secondary invasion by intestinal bacteria could explain the chronic inflammatory changes. Alternatively, decreased resistance to microorganisms caused by the factors discussed earlier could facilitate the invasion of the mucosa by the intestinal bacteria. A third explanation presents itself: Minor degeneratory changes of the crypts were found in the 1st week in animals of all experimental groups and these changes were apparently caused by the irradiation. After a radiation dose of 675 r and treatment with isologous bone marrow, late intestinal changes were never seen. It seems therefore possible that the general debility of the animals treated with foreign bone marrow impeded the recovery of the radiation-induced intestinal damage. Unhealed mucosal defects could then be easily visualized as a portal of entry for intestinal bacteria. Significant in this respect is the finding that severe diarrhea occurred in F_1 hybrids after irradiation with 950 r and treatment with parental bone marrow, while no diarrhea was found in similar experiments when a dose of 675 r was administered (*30*). Intestinal lesions caused by impaired recovery of radiation-induced damage, delayed death, weight loss, and diarrhea have also been described in mice subjected to repeated sublethal radiation doses (*42*).

Congdon and Urso (*22*) reported the occurrence of hemorrhagic lesions of the stomach and intestines—necrotic changes, purulent inflammation, and extensive bacterial growth. Comparable lesions were found in our experiments in the 1st month after irradiation. These can be easily distinguished histologically from the late intestinal lesions described in this paper, and the former are, in our opinion, due to septicemia and hemorrhagic diathesis.

In the liver two distinct lesions have been described. Focal necrosis, which resulted in the loss of all structural elements and often was associated with extensive bacterial growth, was found in the 1st month both in irradiated, untreated mice and in mice treated with foreign bone marrow. This early hepatic necrosis is clearly associated with bone marrow aplasia, and it could be caused by septicemia or hemorrhagic shock.

Late hepatic necrosis characteristically only involved the liver cells. The lobular connective-tissue stroma was preserved and incidentally showed signs of proliferative activity. Possibly the degeneration of the liver cells is a direct consequence of the immunological activity of the graft. Inanition and chronic diarrhea might account for the appearance of the lesion which closely resembles acute yellow atrophy.

Hepatic necrosis in radiation chimeras has also been described by Congdon and Urso (*22*) and by Ilbery, Koller, and Loutit (*23*). The latter

authors suggest that the lesions resemble those found in Tyzzer's disease which is caused by *Bacillus piliformis*. Intracellular proliferation of bacteria in the liver cells at the periphery of the lesion has been described as typical for Tyzzer's disease (*43*). In our material this was never apparent. An infectious etiology cannot be excluded, however, since transfer of the liver lesion by inoculation of liver homogenate into normal mice has not been attempted.

A histological examination of *the skin* has not been regularly performed in these mice. Histological changes of the skin in mice of other experiments are reported elsewhere (*32*). It is likely that the skin changes which appeared after the 2d month have an immunological basis, since similar lesions were induced in nonirradiated mice by the injection of homologous lymph-node cells (*32*). It is of interest that skin lesions were not seen in total reversals.

No simple explanation can be afforded for *the vascular inflammations* observed, in the 1st month, in mice treated with foreign bone marrow. The lesions may be either the result of septicemia or a manifestation of an antigen-antibody reaction. The vascular lesions were seen in the period of maximum incidence of bone marrow aplasia, and in 2 mice a septicemia could be demonstrated. This suggests that the vascular inflammations are of bacterial origin. However, only in 4 of 13 animals with vasculitis was bone marrow aplasia actually present. Furthermore, vascular lesions were never seen in the irradiated, untreated controls. It seems therefore that the development of the lesion is conditioned by the ability of the animal to react to bacterial invasion with cellular exudation, which is possible only in the presence of an at least partially functioning bone marrow. Although vascular lesions of the kidney were never observed, the possibility cannot be excluded that the necrotizing vasculitis is a more direct consequence of a host anti-graft reaction.

Progressive degeneration of the myocardium, resulting in extensive calcification of *the heart*, was frequently observed in mice treated with foreign bone marrow and in the irradiated, untreated controls. Inanition could be directly responsible or it could have prevented recovery from the irradiation.

Histological examination of *the testis* showed that almost complete recovery of spermatogenesis occurs after total-body irradiation with 675 r. Recovery was delayed and irregular in mice treated with foreign bone marrow as compared to those treated with isologous bone marrow. This probably is due to the wasting of the mice in the former group.

Contrary to the pathological changes in the 1st month, which evidently are the result of an immunological reaction of the host against the graft, the symptom complex and the lesions occurring later cannot be readily explained by an immunological mechanism. A number of pathological changes might be at least partly ascribed to the impaired recovery from irradiation effects (colitis, delayed recovery of spermatogenesis, and myocardial calcification).

In our material a prominent aspect of secondary disease has been the presence of multiple, localized inflammatory processes (pneumonia, colitis, dermatitis, and possibly liver necrosis). These lesions could have resulted from decreased immunological resistance against bacteria and are at most indirectly related to the presence of a foreign graft of hematopoietic tissue. It is possible, however, that a number of the lesions are initiated by an immunological reaction of the graft against the host (dermatitis, crypt loss in the large intestine, hepatic necrosis, and atrophy of the lymphatic tissues).

As discussed earlier, atrophy of the lymphatic tissues might be explained by a prolonged antigen-antibody reaction (*22*). Immunological activity of the graft against the host could therefore result in immunological exhaustion. By decreasing the resistance against bacterial infections, this could be the initial lesion which starts the whole chain of events leading to the death of the animals.

Finally, a hitherto undetected lesion or functional disturbance caused by a graft anti-host reaction might be the initial lesion involved in the development of secondary disease. In mice that were treated with large amounts of homologous lymph-node cells and that died acutely between the 6th and 10th days after irradiation, no gross pathological changes could be detected (*32*). Nevertheless the mortality following this procedure has in all probability been due to an immunological reaction of the graft against the host. In order to detect such a lesion, more extensive pathological examination of mice that died of this acute syndrome is needed.

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PLATES

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PLATE 140

FIGURE 1.—Focal necrosis showing total loss of architecture and bacterial growth in liver of male CBA mouse that died 21 days after total-body irradiation (675 r) and treatment with heterologous (rat) bone marrow, 50×10^6 cells. Hematoxylin and eosin. × 192

FIGURE 2.—Regeneration of sternal bone marrow in male CBA mouse killed 5 days after total-body irradiation (675 r) and treatment with heterologous (rat) bone marrow, 50×10^6 cells. Hematoxylin and eosin. × 192

FIGURE 3.—Aplasia of sternal bone marrow in male CBA mouse that died 21 days after total-body irradiation (675 r) and treatment with heterologous (rat) bone marrow, 50×10^6 cells. Hematoxylin and eosin. × 192

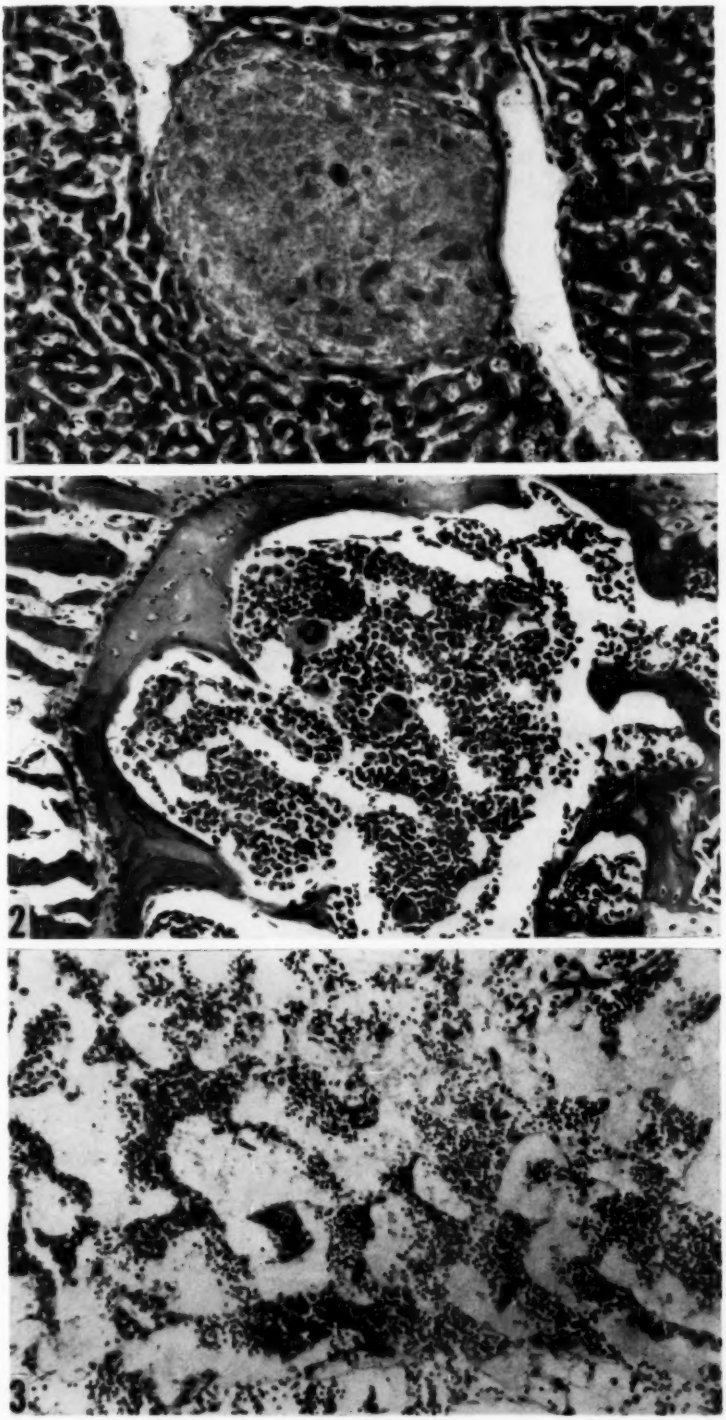
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PLATE 141

FIGURE 4.—Atrophic splenic nodule showing edema and deposition of fibrin-like substance. Extramedullary hematopoiesis in surrounding red pulp. Male CBA mouse killed 44 days after total-body irradiation (675 r) and treatment with homologous bone marrow, 22.5 $\times$ 10^6 cells. Hematoxylin and eosin. $\times$ 192

FIGURE 5.—Mesenteric lymph node showing dense perivascular plasma-cell collections and numerous macrophages. Male CBA mouse killed 15 days after total-body irradiation (675 r) and treatment with heterologous (rat) bone marrow, 50 $\times$ 10^6 cells. Hematoxylin and eosin. $\times$ 192

FIGURE 6.—Necrotizing vasculitis in mesentery of male CBA mouse killed 15 days after total-body irradiation (675 r) and treatment with heterologous (rat) bone marrow, 50 $\times$ 10^6 cells. Hematoxylin and eosin. $\times$ 120

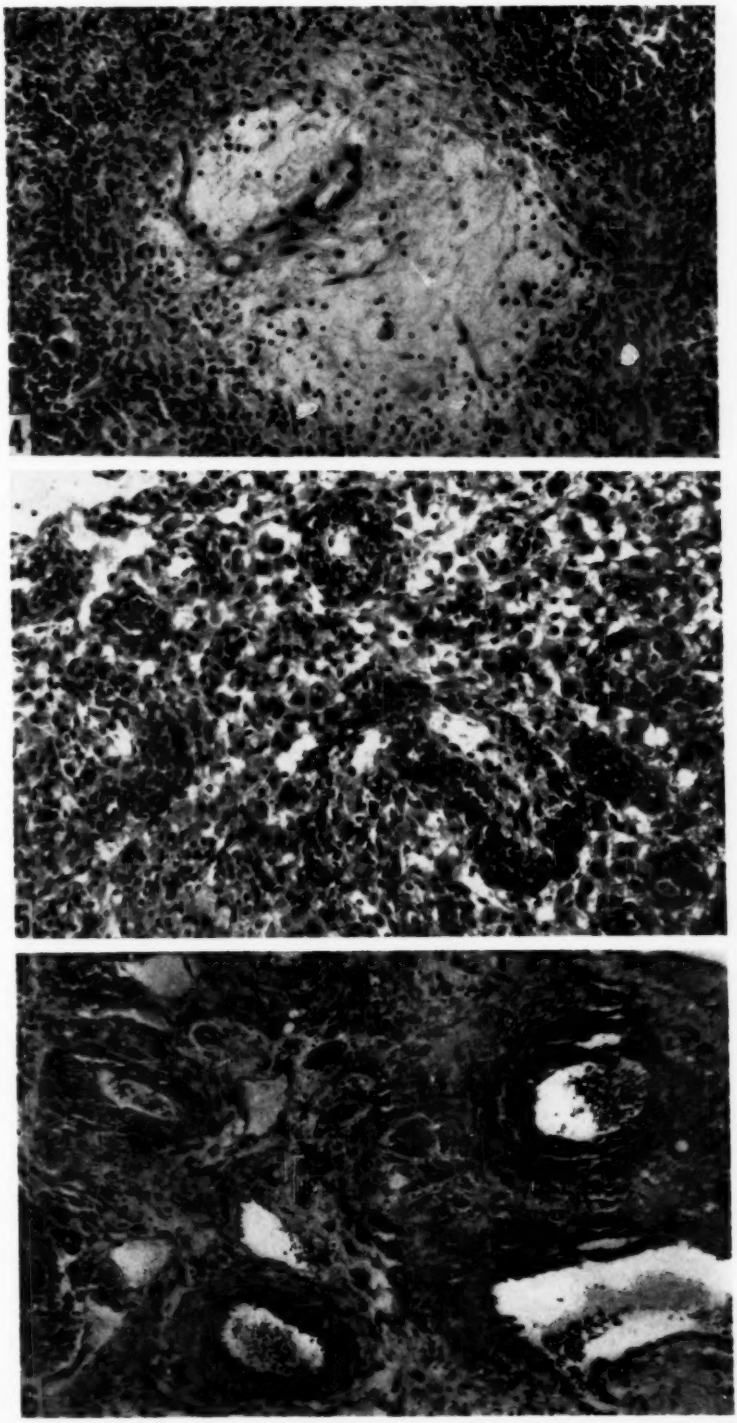

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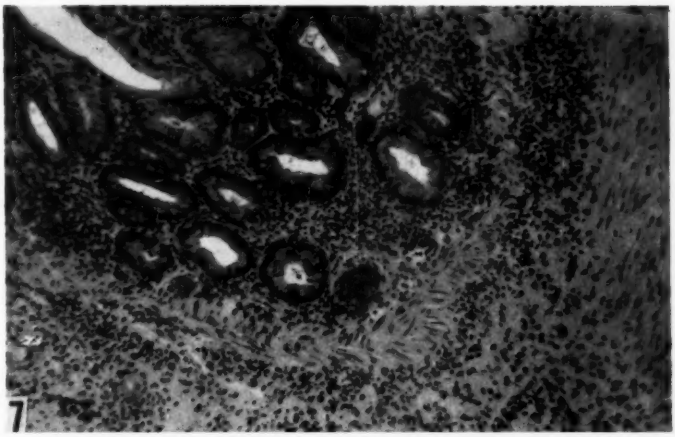
FIGURE 7.—Large intestine showing infiltration of mucosa and submucosa with neutrophils and mononuclear and plasma cells. Edema of submucosa. Male CBA mouse that died 43 days after total-body irradiation (675 r) and treatment with homologous bone marrow, 22.5×10^6 cells. Hematoxylin and eosin. × 120

FIGURE 8.—Crypt degeneration and cellular exudate in mucous membrane of large intestine of a male CBA mouse killed 44 days after total-body irradiation (675 r) and treatment with heterologous (rat) bone marrow, 50×10^6 cells. Hematoxylin and eosin. × 480

FIGURE 9.—Necrosis of hepatic parenchyma with preservation of stromal connective tissue. Vascular dilatation. Male CBA mouse killed 38 days after total-body irradiation (675 r) and treatment with heterologous (rat) bone marrow, 50×10^6 cells. Hematoxylin and eosin. × 120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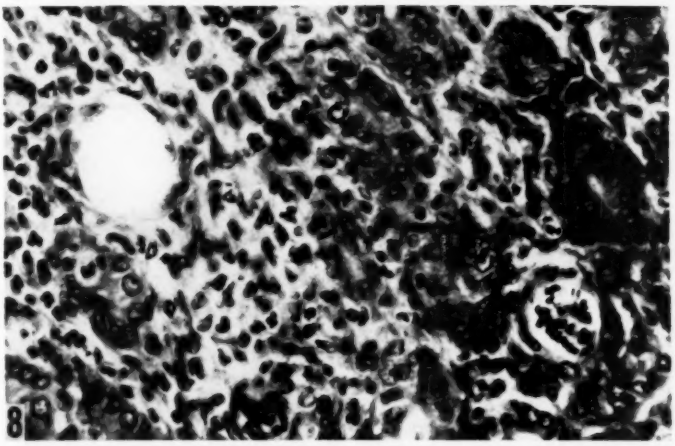
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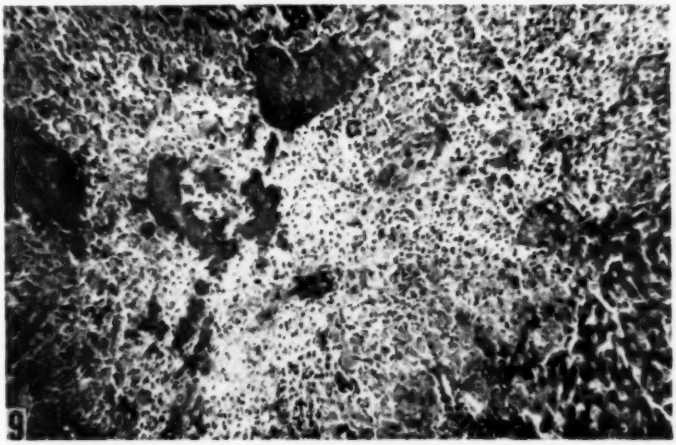
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Oncogenic Effect of Tissue-Culture Preparations of Polyoma Virus on Fetal Mice [1]

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SUMMARY

Swiss mice inoculated late in fetal life with tissue-culture preparations of SE polyoma virus died earlier and had a higher incidence of tumors than animals inoculated shortly after birth. No histologic types of tumors were seen in the mice treated *in utero* that had not been seen previously in mice treated as newborns. Some of the treated mice were the offspring of mothers bred near mice that had been inoculated previously with polyoma-virus preparations. The incidence of salivary-gland tumors and renal lesions was significantly lower in mice from those mothers that had been indirectly exposed to inoculated animals than in mice from unexposed mothers. A comprehensive morphologic analysis of the tumors indicated interrelated patterns of development and behavior which are unique among murine neoplasms. Initially in organs where tumors developed, epithelial cells hypertrophied, formed intranuclear inclusion bodies, and degenerated. The tumors were composed of hyperplastic epithelial elements and proliferating undifferentiated cells that were limited in their capacity to invade adjacent tissues or metastasize. Some tumors regressed, while other caused death of the animal only by local growth in vital organs.—J. Nat. Cancer Inst. 23: 1441–1475, 1959.

THAT A particular variety of neoplasms occurs in mice inoculated with the supernatant fluid of mouse-embryo tissue cultures which contain the SE polyoma virus, a subcellular replicating agent originally recognized in the leukemic tissues of AKR mice (*1*, *2*), has been shown by Stewart, Eddy, and Borgese (*3*). Certain attributes of the host are critically related to the production of the tumors.[5] The maturity of the mice at the time of inoculation is important, since only those inoculated early in life have developed the particular group of tumors characteristic of this phenomenon. Gross (*4*), using cell-free extracts of leukemic tissues from AKR mice, has emphasized that only leukemia develops in several strains

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[5] In this paper *tumor* and *neoplasm* are used synonymously to refer to any abnormal proliferation of cells without regard to the stimulus for proliferation or the ultimate behavior of the cells.

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of mice inoculated more than 16 hours after birth, but the same strains develop a variety of tumors, if inoculated before this time. Since the cells of different tissues proliferate at different rates and differentiate at different times during prenatal and early postnatal periods, this experiment was designed to determine whether mice inoculated *in utero* would develop a different variety of lesions from those that occur in mice inoculated after birth.

During the experiment it was found that tumors in postnatally inoculated mice were more frequent in some litters than in others. Law *et al.* (*5*) had observed the same effect with inbred mice inoculated at birth with extracts of leukemic tissues from strain AKR mice. Aside from the genetic heterogeneity of our mice (a factor not present in Law's experiment), the high and low tumor-incidence litters differed only in that the mothers of the latter group had been housed during breeding and gestation in the same room with animals previously inoculated with polyoma tissue-culture preparations. Efforts were then made to evaluate the various aspects of tumor development in terms of age of the mice at the time of inoculation and the influence of indirect contamination of the mothers.

MATERIALS AND METHODS

Tissue-culture preparations.—Decanted fluids from a series of mouse-embryo tissue cultures, designated subline 3919, were used.[6] Subline 3919 represents one of a group of serially inoculated tissue-culture sublines that produce a particular variety of tumors in mice. The complete history of subline 3919, its relation to other sublines, and the techniques of tissue-culture preparation have been recorded in previous publications (*6*, *7*). The first tissue culture of subline 3919 was inoculated with the extract of a salivary-gland tumor from a $(C3H_t \times AKR)F_1$ hybrid mouse. Each subsequent tissue culture in the series was a newly initiated culture of mouse-embryo cells, which was inoculated with the fluid from the culture immediately preceding it. Decanted fluids from tissue-culture passages 17, 18, 22, 23, and 25, of subline 3919, were used to inoculate fetuses *in utero* and newborn animals. The fluids were kept at 4° C. for 1 to 10 days prior to the inoculation of the animals.

Animals.—Randomly bred Swiss mice, from the Jay-Poiley colony, Animal Production Section, National Institutes of Health, were the parents of all test animals. The mice of this colony have been shown to be free of serologic evidence of the polyoma virus (*8*, *9*).[7]

The mice were received in our animal colony at 1 month of age and

[6] Drs. Bernice Eddy and Sarah Stewart provided the decanted fluids.

[7] Serologic tests for antibodies to polyoma virus had not been perfected at the time this experiment was carried out. Since then, Eddy *et al.* (*10*) and Rowe *et al.* (*11*) have developed hemagglutination-inhibition tests and complement-fixation tests, which show high titers by the 4th month of life in a high proportion of mice exposed by various direct routes to polyoma virus tissue-culture preparations or excreta from treated mice (*9*). Untreated mice of 3 colonies have shown a high incidence of serologically positive mice; however, mice of the Jay-Poiley colony have remained negative (*9*).

encouraged to breed by housing 5 females and 1 male in small plastic cages, with perforated tops. During the breeding months and during gestation, the breeders were housed adjacent to cages of mice that were being inoculated with polyoma virus or that had tumors resulting from such inoculations. The females were observed daily for vaginal plugs and, when obviously pregnant, were transferred to individual cages. The fetuses of 16 gravid females were inoculated. The fetuses represented either the first or second litter of females that were 4 to 5 months old. Neither the mothers nor their previous offspring had been inoculated with polyoma virus. Nine of the 16 pregnant mice either died or resorbed all their fetuses. Most pregnant females that resorbed their fetuses were inoculated before the 17th day of gestation. The remaining 7 mothers carried 55 treated fetuses, but gave birth to only 43 young. Table 1 indicates the time during gestation when the fetuses were inoculated, and the survival rate of the 7 litters at birth. These 7 litters from mothers housed near virus-inoculated animals are designated the fetal-inoculated group.

TABLE 1.—Survival rate, at birth, of 55 mice inoculated 1 to 4 days before birth—normal gestation time 18 to 22 days

Litter No.	Time between vaginal plug and delivery	Days between inoculation and birth	Live births / Number inoculated
93	17 days	2	4/8
96	18 days	1	5/7
99	?	1	8/8
102	21 days	4	6/7
103	20 days	1	8/8
91	?	2	8/9
92	21 days	1	4/8

As comparable controls, the 12- to 24-hour-old first-born litters of 4 other females from the same cages were caged separately with their mothers and were inoculated with the same or subsequent passages of polyoma culture subline 3919. These 4 litters inoculated as newborns are designated the contaminated-control group, since the mothers also had been housed near previously treated animals. A third group of 3 litters served as controls on both the effect of fetal inoculation and maternal contamination. The 3 litters were born in the virus-free Jay-Poiley colony and inoculated with the tissue-culture preparations within the first 24 hours after birth. Only after inoculation were they housed in the vicinity of contaminated animals. These 3 litters are designated the uncontaminated-control group.

Inoculations.—Under ether anesthesia, a 2-cm. incision was made in the low midline of the mother's abdomen and both uterine horns were delivered from the abdominal cavity. The fetuses were visualized through

the uterine wall and counted. Each animal was inoculated by penetrating the uterine wall and fetal skin with a 30-gauge needle attached to a syringe of 1-ml. capacity and by injecting 0.03 ml. of tissue-culture fluid. An effort was made to inject the inoculum into the soft parts of the hind quarter; however, gross visualization did not always insure that this was the exact site of injection. The technique did insure that all the fetuses were exposed to the inoculum, though some leakage must have occurred from the puncture wounds in the skin. The newborn litters received an inoculation of 0.10 ml. of culture fluid in the posterior cervical and occipital subcutaneous tissues, through a 22-mm., 26-gauge needle. The point at which the needle penetrated the skin, 2 cm. caudal to the inoculation site, was compressed after inoculation to retard leakage. Studies in our laboratory with tissue-culture inoculums colored with the fluorescent dye acridine orange have indicated dissemination of the dye component of the fluids to all areas of the subcutaneous tissues, salivary glands, thymus, lungs, kidneys, and adrenal glands, within 30 minutes. In less that 8 hours all organs of the newborn animal were colored by the dye.

Pathological techniques.—Complete autopsies were performed on all animals that could be salvaged. All animals inoculated as fetuses had died by the 8th month, except one. Animals living 8 months or longer were killed. Cannibalization and *post-mortem* autolysis sometimes precluded a complete histologic study, and deficiencies in examining the commonly affected organs are indicated in the tables. Tissues were fixed in 10 percent formalin, and bones were subsequently decalcified in formic acid-sodium citrate solution. Tissues were routinely stained with hematoxylin and eosin, and selected tissues were colored with Masson's trichrome stain, Lendrum's phloxin-tartrazine stain, phosphotungstic acid hematoxylin, periodic acid-leucofuchsin, and Feulgen's method for DNA. All histologic methods were the same as those described in a recent text (*12*).[8]

RESULTS

The duration of life in mice surviving the newborn period, and the incidence, morphology, and extent of 11 different primary proliferative lesions were compared in the 3 groups of mice.

The 11 lesions, which are listed in the footnote for table 2, commonly develop in Swiss mice inoculated soon after birth with the polyoma tissue-culture preparations (*3*, *13*). The incidence of the 11 lesions and the mortality of the mice in the 3 groups indicated quantitative differences in response (tables 2 and 3). However, no type of tumor was found that had not been previously observed in mice inoculated shortly after birth. The tables do not distinguish between the earliest proliferation of cells in a given organ and more pronounced forms of neoplasia. It

[8] Mr. Joseph M. Albrecht and associates prepared the tissues for histologic examination.

TABLE 2.—Distribution of lesions in Swiss mice inoculated during fetal life with polyoma virus cultures (subline 3919). Mothers of these mice were bred near mice previously inoculated with polyoma virus cultures*

Time of death	Subline 3919, culture 17		Subline 3919, culture 18			Subline 3919, culture 23	
	Litter 93	Litter 96	Litter 99	Litter 102	Litter 103	Litter 91	Litter 92
Less than 48 hours	2 mice cannibalized			3 mice cannibalized	1 mouse cannibalized		
Month 1	0B00000H000 0B00000H000		–B–––––0–––	0 B000000I00 0 B000000000		––––––––––	
Month 2		0 BC000GH0J–					
Month 3		AB00E0GHI–K 0 BC0E0G00––	ABC0E0–HI00	ABC000GH000	ABC000G00J0	AB00E0GHIJ0 AB00E0GH0–– – B00E0 0H0J0 0 BC0E0G00–– ––––––––––	ABC000GH0JK
Month 4		ABC000GH00–	ABC0E0GHIJK ABCD00GHIJ0 ABC000GH000		– BCD00GH0–0	ABC000–H0–0	ABC0E0GH––0
Month 5			ABC000GH0––		ABCD00G–––– – B––––– H––––		
Month 6			0000000000000 0000000000000		A0C000G0000 0BC000G#00– –0 C000–0–00	A0C–00G000–	
Month 7							000000G0000 0–00–00–0––
Month 8 (killed)		00000000000					

*The code letters are symbols for the lesions found in a single mouse:

A: Tumors of the salivary glands and pharyngeal glands.
B: Proliferative lesions of renal tubules.
C: Tumors of the pleura and peritoneum.
D: Tumors of the thymus.
E: Tumors of the breast.
F: Tumors of subcutaneous tissues.
G: Tumors of bone.
H: Tumors of the renal mesenchyme.
I: Proliferative lesions of hair follicles.
J: Tumors of the thyroid.
K: Tumors of the adrenal medulla.
#: Sclerosed tumors of the renal mesenchyme.
+: Sclerosed salivary-gland lesions of doubtful origin.
0: A zero indicates that no tumors were found in the site listed.
–: A dash indicates that examination of the site listed was incomplete.
A: **Bold** letters indicate the most likely source of intravascular pulmonary deposits.

TABLE 3.—Distribution of lesions in Swiss mice inoculated within 24 hours after birth with polyoma virus cultures (subline 3919)*

Time of death	Uncontaminated-control group: mothers bred in colony showing no serologic evidence of polyoma virus			Contaminated control group: mothers bred near mice previously inoculated with polyoma virus			
	Subline 3919, culture 25					Subline 3919, culture 22	
	Litter 116	Litter 118	Litter 120	3919–17 Litter 95	3919–18 Litter 60	Litter 85	Litter 90
Less than 48 hours			5 mice cannibalized				5 mice cannibalized
Month 1							
Month 2							
Month 3	ABC0D00 H0–0			0 BC0E000000			
Month 4	ABC000GH0J0 AB000000000	ABC000000–0	ABC000G00J–			–0C00FG#0–0	
Month 5		ABC00000000	ABC0D0G00–0				
Month 6	A00000000J0 A0000000000	ABC000G0000		000000GH000 00C000–00––			
Month 7		A0000000000		000000G#000 00000000000			
Months 8 to 10 (killed)	+0000000000 00000000000	00000000000 00000000000 00000000000	00000000000	00000F00000 00000000000 00000000000	00C000G0000 +0000000000 00000000000	00C000GH000 A0C000G0000 00000000000 00000000000 00000000000	00000000000

*See footnote table 2.

was therefore considered important to make a detailed morphologic study of the tumors to determine whether such variations might be significant. No attempt was made to describe the complete natural history of the tumors, though animals that died early or late in the experiment permitted certain observations pertinent to this problem.

Morphology of Lesions

Early Degenerative Lesions

Six mice that were inoculated *in utero* and that died between the 9th and 17th days after birth were the youngest mice examined. Five of these had lesions in the convoluted tubules of the kidney, which consisted of hypertrophy and degeneration of the tubular lining cells, with the formation of intranuclear inclusion bodies. This complex of cellular changes is described in detail under the heading "Lesions of the Renal Tubules." Although not tabulated in the list of proliferative lesions (tables 2 and 3), a few examples of similar nuclear alterations and cell degeneration could be found in the cells of the pulmonary alveoli and bronchi, hair follicles, salivary glands, thymic reticulum, and mammary ducts of the very young mice. Similar observations have been previously reported (*3*, *14*). The nuclear changes were associated with cytolysis and, in the lungs, with leukocytic infiltrates. Consistently, mice that reached maturity had intranuclear inclusions in the renal tubules and hair follicles, if cell proliferation was also present in these organs, and a few mice had intranuclear inclusions and hypertrophied cells adjacent to proliferative lesions of the thyroid and thymus. None of the cells that showed clear evidence of having proliferated to form definite tumor masses were affected in this way. The cytologic changes are illustrated in figures 6 to 9, 14, 25, and 27.

Tumors of the Salivary Glands

The morphologic features of the tumors in all major salivary glands were essentially the same as those described by Law *et al.* (*5*). The parotid, exorbital lacrimal, submaxillary, and sublingual glands all had similar histologic lesions, and the frequency in which they were affected was in the order named. The parotid tumors far exceeded the others in incidence and extent of glandular involvement. The submucosal glands of the nasal passages, pharynx, and trachea were involved by smaller tumors of the same type.

The most characteristic features of the tumors were their multicentric origin and growth as separate circumscribed nodules within a single gland. Variations in the morphology of the nodules correlated best with differences in size, and frequently nodules of different size could be found in the same gland. Lesions of microscopic size were seen in old as well as young mice. The smallest lesions were characterized by foci of hyperplastic intercalated ducts, surrounded by cells with nuclei similar to those of duct cells, but with scant cytoplasm. The less-differentiated cells grew in diffuse or centrifugal patterns around the ducts, and all acini were obliterated. Proliferating cells in the smallest lesions sometimes had a loose edematous, intercellular matrix, but more often the cells were crowded together into compact masses which even obliterated cytoplasmic features (fig. 1). Larger nodules were composed of compact fascicles of bipolar spindle cells that were intimately associated with both collagen fibers and areas of mucoid degeneration. In some tumors, transitions were found between the small cells around the hyperplastic ducts and the predominating spindle cells (fig. 2). Recognizable epithelial elements in such tumors consisted of residual ducts, which seemed to have been pushed to the periphery of the nodules by intervening fusiform cells. Some nodules were composed entirely of dense collagen, except for the persistent hyperplastic ducts around the periphery (fig. 4). Medium-

sized nodules often had central zones of cell degeneration. Certain tumor nodules that were exceptionally large, measuring up to 35 mm. in diameter, had a different cellular structure. In these tumors, cells resembling the undifferentiated cells of the smallest tumors obliterated all tubular structures and formed closely packed cell masses permeated by large vascular lacunas (fig. 3). Masses of tumor cells protruding into the vascular spaces often gave the tumors a pseudopolypoid structure. In spite of their large size, these tumors retained a nodular configuration and could be easily separated from surrounding structures. There was no evidence that the cells of tumor nodules metastasized to regional lymph nodes or infiltrated surrounding tissues, but connective tissues at the margins of a few tumors showed remarkable foci of proliferation. These mesenchymal lesions could not be considered invasive growth from the salivary-gland tumors, since they were completely unrelated cytologically to the juxtaposed nodules.

No differences could be detected in the tumors from mice inoculated *in utero* and those from uncontaminated-control mice in respect to size, extent of organ involvement, or morphology. The single parotid tumor observed in the contaminated-control mouse was a large tumor composed entirely of poorly differentiated spindle cells. Two mice inoculated *in utero* and 4 uncontaminated-control mice had metastases confined to the lumens of pulmonary arteries. The primary tumors in these mice were of the massive pseudopolypoid variety in which tumor buds protruded into vascular lakes. It appeared that the pulmonary vessels simply held emboli of viable tumor cells, with a limited capacity for growth.

Lesions of the Renal Tubules

A complex of degenerative and proliferative changes was observed in the renal cortex of some mice from all 3 groups. In immature mice the lesions were characterized by cellular hypertrophy of focal areas of epithelium in the distal tubules. Subsequently, both proliferation and degeneration of cells of the renal tubules were observed in the same areas of affected kidneys. In the hypertrophied cells, nuclear changes were especially notable. The diameters of affected nuclei were 2 to 4 times larger than unaffected nuclei. Within the nuclei a variety of presumably sequential alterations occurred. Most of the enlarged nuclei contained heavy strands and granules of chromatin scattered in a clear nucleoplasm. Some strands of chromatin were attached to nucleoli. In other hypertrophied cells, a central core of nucleoplasm containing homogeneous material seemed to compress the chromatin and the nucleolus against the nuclear membrane. The material in the center of the nucleus was poorly stained by the methods used, but its substance could be recognized by its slight affinity for most basic dyes (fig. 7). A third intranuclear change was noted in most mice, but only in a few of the hypertrophied cells. Nuclei with remnants of the central homogeneous mass contained single, dense, acidophilic inclusion bodies (figs. 6 and 9). The intranuclear inclusion measured up to 6 μ in diameter, and it conformed in shape to the distended, sometimes irregular, outline of the nuclear membrane. The margin of the inclusion was fuzzy in some cells and sharply defined in others, but it was always separated from the nuclear membrane and the marginated chromatin by a clear halo (fig. 9). Unaffected nucleoli could be recognized in the marginated chromatin of inclusion-laden cells. The Feulgen reaction was negative, and the periodic acid-leucofuchsin reaction was faintly positive in both recognizable inclusion bodies and the homogeneous nuclear material which seemed to precede the inclusion. The inclusions were stained intensely with phloxin by Lendrum's technique (*15*). Circumscribed masses of similar phloxinophilic inclusion particles could be found in the cytoplasm of cells showing karyolysis and in the lumens of tubules lined by degenerated cells (fig. 8). Degenerative lysis of cell membranes and nuclear pyknosis were common in affected cells. In pyknotic cells, a rim of chromatin adhered to the inclusion. In the animals that died during the first 4 months, the number of cells with altered nuclei was roughly proportional to the number of cells in advanced

stages of degeneration, which suggested that nuclear changes frequently resulted in death of the cell. However, separate tubular lesions in the same kidney often showed different degrees of involvement, and did not always show simple degenerative end results. In many kidneys, areas of hypertrophied lining cells and areas of renal tubular cells that had actively proliferated were closely associated. Proliferation was indicated by linings of crowded basophilic, columnar cells with pseudostratified arrangement due to nuclear disorientation (figs. 5 and 6). Mitotic figures were common in these hyperplastic cells, but the previously described abnormal intranuclear changes were not observed. Proliferation of cells in the more overt lesions formed papillary folds which protruded into dilated tubular lumens, but none of the lesions showed cellular invasion through the basement membrane of the tubule. The tubular lesions were also associated with nodular, perivascular infiltrates of mononuclear leukocytes. The infiltrates differed only in degree from those seen in other diseases of the mouse kidney.

The lesions of the renal cortex were qualitatively similar in the three groups of mice. Lesions in the tubules of older animals were predominantly proliferative, though persistent hypertrophied cells containing intranuclear inclusions often could be seen in single cells, sometimes isolated from proliferative lesions. Tubular lesions were more pronounced in the fetal-inoculated group at all ages, while leukocytic infiltrates were more characteristic of older animals inoculated during the first 24 hours of life.

Proliferative Lesions of the Mesothelium

Minute warty excrescences were seen on mesothelial surfaces, frequently on the pleura, but they also involved the pericardium and peritoneum, in some animals. The earliest lesion, noted in a 2-month-old mouse inoculated *in utero*, consisted of a focus of hypertrophied mesothelial cells above a thickened hyalinized matrix (fig. 12). The more developed lesions were multiple and composed of filiform fibrovascular stalks attached to the visceral surface by narrow pedicles (fig. 11). These finger-like villi were covered by 1 to 5 layers of cuboidal mesothelial cells. Although the lesions sometimes covered the entire serosa and formed polypoid masses up to 2 mm. in diameter, the surface layers of mesothelium rarely showed mitotic figures and they retained uniform cellular arrangements. The 33 examples of the lesion studied did not metastasize or invade underlying tissues.

Regressive changes were more apparent in this lesion than in others. Regression was indicated by transitions from hyperplastic, cellular polyps to densely sclerotic, filiform projections on the pleura that were covered by a simple layer of flattened mesothelial cells (fig. 10). Avascular hyalinized mesothelial tags of this type were seen commonly as single lesions in animals that survived 7 months or longer. It is possible that these lesions regress completely without leaving a scar, since the attachment to the visceral surface was a thin pedicle that did not involve the parenchyma. Yet the lesion was the most common in the treated animals, occurring in the absence of all other lesions, and thus it may represent the most sensitive response in the Swiss mouse. The lesions in the fetal-inoculated mice were generally larger and had more proliferating cells than lesions in the mice inoculated as newborns, but no structural differences in lesions of the 3 groups were evident.

Tumors of the Thymus

Seven mice from the fetal-inoculated group showed changes in the thymus. Four mice had lesions consisting of disorganization of the corticomedullary architecture secondary to focal loss of cortical thymocytes and proliferation of the cortical reticular cells. Some hyperplastic reticular cells were in mitosis, while others had atypical vesicular nuclei with prominent nucleoli. In one mouse, intranuclear inclusions were seen in the hypertrophied reticular cells. The three fetal-inoculated mice listed in

table 2 had tumors grossly apparent as circumscribed masses that developed in atrophic glands. Each tumor was less than 3 mm. in diameter and showed no evidence of invasion or metastasis. The cells composing the nodular growths were reticular, sparsely infiltrated with thymocytes, and within such areas cuboidal epithelioid cells formed adenomatous patterns of growth. In one thymic tumor the epithelioid cells merged with an area of organoid glandular structure in which atypical glands were supported by a loose mesenchymal stroma and lined by simple columnar epithelium with ciliated free borders (fig. 13). The glandular structures are probably related to cysts, sometimes lined with ciliated epithelium, that occur in untreated mice (*16*). Both structures may represent divergent differentiation of the framework of thymic reticular cells that are derived from entoderm of the pharyngeal pouches.

Tumors of the Breast

Mammary tumors occurred in 12 of the 72 treated animals that survived the newborn period. All the tumors except one occurred before the 5th month. In the fetal-inoculated group, 3 occurred in male mice; grossly the tumors were located in the axillary, cervical, or inguinal regions. There were multiple tumors in 4 females. Reference has been made previously to the hypertrophied cells and nuclear inclusions in the mammary ducts of a mouse that died at 13 days of age (fig. 14). The subsequent lesions studied were fully developed tumors, measuring 10 to 45 mm. in diameter, that did not show such changes.

Histologically, all the tumors showed similar structural patterns. New growth of duct epithelium, with the formation of distorted cysts containing papillary and adenomatous formations of hyperchromatic epithelial cells, was seen in most tumors. However, compact growth of less differentiated cells between the ducts generally overshadowed recognizable adenomatous proliferation. In more overt form, this growth pattern resembled massive intraductal papillomas in which the stroma was formed of compact masses of undifferentiated cells. The remarkable uniformity in cell structure and in organization of the stromal growth was in contrast to the usual disorderly cell structure of medullary carcinomas (fig. 15). The tumors did not fit any major morphologic type of mouse mammary tumors described by Dunn (*17*). Their structural development seemed analogous to that of the salivary-gland tumors. However, recognizable glandular components of the two tumors were quite different, and the medullary components of the mammary tumors did not show the mesenchymal variations noted in salivary-gland tumor nodules.

There were no qualitative differences in the tumors of the 3 groups of mice. Five fetal-inoculated mice and 1 mouse in the uncontaminated-control group had intravascular tumor deposits in the lung, presumably from the breast tumors.

Tumors of the Subcutaneous Tissues

Aside from the proliferation of connective tissue adjacent to the salivary-gland nodules, tumors of the subcutaneous tissues of the inguinal region were found in 2 mice of the contaminated-control group. The tumors occurred in 4- and 8-month-old mice. The tumor in the younger mouse was a cavernous hemangioma; in the other it was a spindle-cell tumor which invaded adipose tissues. Although both Gross (*18*) and Dulaney (*19*) have reported significant increases in subcutaneous tumors after somewhat similar treatment of newborn strain C3H mice, the fact that such tumors can occur spontaneously in some strains (*20*) would indicate that no particular significance can be attached to the very low incidence in our groups of randomly bred animals.

Tumors of the Bone

Osteogenic tumors have been reported to occur in 22 percent of mice that developed tumors after inoculation with similar preparations (*14*). However, only brief refer-

ence has been made to the nature of these lesions (*3*, *5*). In this study bone tumors were found in 21 fetal-inoculated mice and in 10 postnatally inoculated mice. Our record of incidence is not complete since many of the lesions were of microscopic size, and routine microscopic examination was done only on the vertebrae and skull. The lesions were usually multiple. The cranial bones, especially those of the occipital region and those bordering respiratory passages, like the maxilla and nasal-turbinate bones, were the most commonly affected in all groups. The vertebral bodies, ribs, shoulder girdle, pelvis, and femur were involved less frequently. The size, extent of cell proliferation, and amount of intercellular matrix of a lesion gave a rough impression of its activity. The lesions ranged from microscopic proliferations of the periosteum through sessile osteophytes to massive cellular tumors that penetrated adjacent soft tissues and, in one mouse, metastasized. Most tumors lay between these extremes and seemed to represent variations of a single process. All these variations might be found at different sites in a single animal.

The smallest recognizable changes were not unlike a reactivation of the normal process of osteogenesis, though seemingly irregular and uncontrolled. A continuous layer of hyperplastic cuboidal cells lined periosteal and, rarely, endosteal surfaces, which sometimes could be detected only by noting the focal and asymmetrical distribution of the lesions (fig. 17). At the time of examination, the hyperplastic osteoblasts did not show nuclear alterations or degenerative changes, though such changes may have occurred earlier. Further proliferation of the periosteal cells resulted in the formation of a multicellular layer of spindle-shaped cells separated from the cortex by intercellular deposits of osteoid ground substance (fig. 18). Such lesions extended over wide areas of the cortex in the form of slightly thickened plates of partly calcified osteoid. In cartilaginous bones, the tumors were usually unrelated to zones of enchondral ossification, though these zones could be affected.

Some lesions, especially those in old mice, contained few cells and appeared to proceed no further than to form thickened cortical or intermedullary foci of osteosclerosis. In other lesions it was apparent that less restricted growth had formed large osteophytic masses, but again these tumors were often composed of massive deposits of osteoid with only a few scattered nests of trapped osteoblasts and a thin margin of spindle cells (fig. 19). It appeared that these tumors had been growing very slowly or had ceased growing. In other tumors, continuing growth along irregularly scalloped margins extended into adjacent soft tissues. In the vertebra, tumors of this type often caused death by extension into the vertebral canal and by compression of the spinal cord (fig. 16). The margins of the more active tumors were composed of irregular, thick masses of spindle and polygonal cells in which rare mitoses were seen. Behind the advancing tumor edges, bars of osteoid were deposited between the cells, isolating cords of tumor cells that trailed from the periphery. Early deposits of osteoid were surrounded by palisades of spindle cells. In the deeper parts of the tumors, massive deposits of osteoid trapped single tumor cells, and the matrices were partially ossified. The single tumor that metastasized to the lung was largely composed of proliferating cells with only scant areas of osteoid, though osteoid deposition was clearly evident in the metastasis.

The more rapidly growing tumors sometimes contained different types of cells. Scattered multinucleated cells resembling osteoclasts were often observed near islands of osteoid deposition. At the periphery of some tumors neoplastic fat cells were seen. Proliferating lipoblasts and lipocytes interspersed with mononuclear leukocytes resembled bone marrow, though no unequivocal areas of hematopoiesis were found. Fat cells formed a large part of two tumors that apparently arose from the cortices of long bones. Both tumors were formed of proliferating lipoblasts in which islands of osteoid-forming cells were prominent (fig. 20). A third variant, not commonly seen, consisted of cells that resembled poorly differentiated chondroblasts. A basophilic acellular matrix, which differed from the osteoid material in adjacent areas, surrounded individual cells and formed cartilagenous islands in the tumors.

The mice in the 3 groups could not be distinguished by differences in the osteogenic

tumors that developed, but the few tumors in mice of comparable age in the fetal-inoculated group were more extensive than those in mice inoculated as newborns. Only one tumor (from the vertebrae of a 9-month-old mouse) in the contaminated-control group attained the size and showed cell proliferation as great as that seen in over one half the tumors in the fetal-inoculated group.

Tumors of the Renal Mesenchyme

Tumors of the renal papilla were found in 20 mice of the fetal-inoculated group and in 6 mice of the postnatally inoculated groups. The lesions were first observed between the tubules that traverse the medulla as minute proliferations of fusiform mesenchymal cells associated with fine collagen fibers. Multiple foci of growth were common within a single kidney and were located either at the corticomedullary junction or in the papilla. In either location, the tumors were unrelated topographically to the renal tubular lesions, though the latter were invariably present. As the tumors became larger, the direction of growth was toward the renal pelvis. The largest tumors, which sometimes exceeded 6 cm. in diameter, were observed in mice with unilateral tumors. They were composed of blunted polypoid tumor masses that projected into hydronephrotic sacs formed by the renal pelvis (fig. 21). Even in the massive tumors, the capsule of the kidney remained intact along with residual areas of cortex. The cellular morphology did not change remarkably with increases in size. Mitotic figures were common in some tumors and rare in others, but in all tumors the tissue was formed of irregular bundles of undifferentiated spindle cells. Blood vessels within the tumors frequently showed hyperplastic endothelial and muscular linings (fig. 22). Accumulations of collagen, which obliterated wide segments of tumor tissue, could be found even in areas with abundant capillary vasculature. Sclerosis was not always present in the larger tumors; in fact, the small tumors of old animals showed the greatest scarring. Three animals had nodular lesions of papillae, which were composed almost entirely of scar tissue with only a few undifferentiated cells at their margins (fig. 23). These are designated in the tables as regressed tumors. Oddly, the only animal in the group with a metastasis from a renal tumor was one of these 3 mice. The solitary lung metastasis was also partially sclerosed.

Again it was evident that the 3 groups of mice could not be separated by any particular feature within the tumors. Yet 15 of the 20 tumors in the mice of the fetal-inoculated group were bilateral, and massive involvement of both kidneys seemed directly responsible for 11 early deaths. None of the 6 tumors in the control groups were bilateral, though unilateral involvement in 2 mice apparently permitted the tumors to grow to sizes comparable to the largest tumors in the fetal-inoculated group.

Lesions of the Hair Follicles

Lesions of the hair follicles did not attain the large size described in experiments with other polyoma-virus preparations (*3*). However, routine examination of the skin revealed 6 examples of early or abortive lesions in the fetal-inoculated group. The earliest lesions observed were in the hair follicles of an 11-day-old mouse that also had intranuclear inclusions in the lungs and salivary glands. The other 5 mice had lesions in follicles of hair and vibrissae in the skin overlying cranial bone tumors or breast tumors (fig. 24). Lesions in the youngest mouse consisted primarily of necrotic follicular cells in the hair bulb. Those cells that survived were markedly hypertrophied and frequently contained nuclei with vacuoles and intranuclear inclusions somewhat like those of the renal tubular cells (fig. 25). The inclusions differed slightly in that they were multiple and more variable in size, and all had smooth spheroidal outlines. Inclusions were present in all lesions, but the predominant alteration in the larger lesions was a proliferation of all layers of the base of the follicle. Such lesions consisted of dilated, tortuous sacs lined by disorganized layers of epithelium 10- to 30-layers thick, with a residual core of desquamated epithelium and keratin serving only to

identify the structure. In these microscopic lesions the proliferative response was confined to the lower half of the follicle and did not involve the neck of the follicle. Neither did it involve the adjacent surface epithelium nor the epithelium of sebaceous glands. The lesions were delimited on all sides by a residuum of elastic tissue which appeared to restrict the proliferating cells to form tubular structures. That these lesions also could regress was indicated by enlarged cystic follicles that were devoid of cells and contained only a shell of desquamated eosinophilic debris.

Tumors of the Thyroid

Proliferative lesions were found in the thyroid glands of 7 fetal-inoculated mice and 3 mice from the uncontaminated-control group. In 3 of the youngest mice in the fetal-inoculated group, the lesions consisted primarily of multiple, circumscribed foci of follicles that were smaller than the surrounding follicles and were depleted of colloid. The affected follicular cells were intensely hyperchromatic and crowded together. Occasionally follicle cells outside these areas contained vesicular nuclei and intranuclear inclusions similar to those described in renal tubular epithelium. Both degenerating cells and cells in mitosis were present in the hyperplastic follicles. The lesions in older mice consisted of similar areas of follicular cell hyperplasia, but proliferating cells, which were smaller and less differentiated, occurred outside the affected follicles. Sometimes growth of cells between the follicles obliterated the glandular pattern and formed a solid tumor of undifferentiated polygonal cells. More commonly, however, glandular proliferation formed circumscribed adenomatous nodules (fig. 28). Larger tumors of this type were delimited by a thin band of vascular connective tissue. The interstitial growth of anaplastic cells, accompanied by papillary proliferation of cells lining the follicles, resembled the growth pattern in tumors of the salivary glands and breast. Infiltrates of mononuclear cells were seen between the glandular components of some of the tumor nodules. The only tumor that was grossly apparent measured about 2 mm. in diameter. Unlike the smaller lesions, this tumor was composed entirely of papillary and glandular structures lined by single pseudostratified layers of small hyperchromatic epithelial cells (fig. 26). Mitotic figures were frequently observed. The tumor was encapsulated by a thin layer of fibrous tissue. Follicles outside the fibrous wall were lined by hypertrophied cells with intranuclear inclusions (fig. 27), but the tumor cells did not show such changes. This tumor occurred in a 4-month-old mouse, from the fetal-inoculated group, that had all but 2 of the 11 described lesions.

Tumors of the Adrenal Medulla

Three tumors were observed in the adrenal glands of the fetal-inoculated group; they were unilateral and similar to those observed in mice inoculated with filtrates of leukemic tissue from ($C3H_f \times AKR)F_1$ mice (*21*). There was no doubt that the tumor in the youngest mouse developed from cells of the medulla, since serial sections indicated that it was confined to the medullary zone (fig. 29). Structurally the cells of this lesion *in situ* closely resembled unchanged medullary cells, though they showed no chromaffin reaction after mordanting for 4 days in Orth's fixative. The cells differed from normal medullary cells in their more basophilic cytoplasm, larger nuclei, and loss of structural orientation. Mitotic figures were remarkably numerous, and tripolar metaphases were observed. Ten of 1,000 tumor cells were in stages of mitosis later than prophase. Two hundred of the 1,000 cells had nuclei in which all the chromatin was aggregated into thickened chromosome bars, which were distributed along the periphery of intact nuclear membranes. Transitions between these altered forms and recognizable spiremes indicated that they represented early mitotic prophase. This high incidence of cells in one phase of mitosis suggested a recent, simultaneous burst of mitotic activity, or of mitotic arrest at this stage of division (fig. 31).

The 2 adrenal tumors in the older mice were much larger, measuring 6 and 8 mm. in

diameter. Both tumors destroyed all the cortex except marginal strips, though the adrenal capsules remained intact. The pattern of growth indicated that the cortex was destroyed by compression rather than by invasion (fig. 30). The tumors were formed of loose cords of basophilic polygonal cells like the cells of the tumor *in situ*. The cells were occasionally in mitosis, and neoplastic giant cells were present. In addition, the tumor contained fibrovascular trabeculae formed of collagen-rich syncytial nests of proliferating anaplastic cells with small fusiform nuclei unlike the cells that formed the bulk of the medullary tumor (fig. 32).

No adrenal tumors were noted in the 35 mice inoculated as newborns. However, previous reports (*21*) indicate that metastasizing transplantable tumors of the adrenal do occur in mice after postnatal inoculation with similar preparations. Jones and Woodward (*22*) refer to a number of adrenal medullary tumors that have been observed in untreated mice, and report their occurrence in 3 litter mates of hybrid (C3H $\times$ I)F_1 mice. There is some indication from their descriptions that the previously reported tumors were unlike those observed in our mice.

Tumors of the Lung

The only other tumors noted in this series of animals were alveologenic pulmonary tumors of the type commonly seen in mice (*23*). Single tumors were observed in 2 of 9 fetal-inoculated mice and 5 of 20 newborn-inoculated mice that survived 6 months or longer. We did not include this tumor as a measure of induced response, since this incidence is no greater than that which can occur spontaneously in our random-bred Swiss mice.

Comparison of the Oncogenic Response of the Three Groups of Mice [9]

Since factors other than the treatment influence survival during the newborn period, only those animals that survived the first 48 hours of life were used to determine the effect of inoculation on lifespan. The median survival time for the 37 fetal-inoculated animals was 3 months. At this time 17 of 18 contaminated-control animals and 16 of 17 uncontaminated-control animals were still alive. At the end of 7 months, all but 1 of the 37 fetal-inoculated group had died, and 12 of 18 mice in the contaminated-control group and 6 of 17 in the uncontaminated-control group were still alive. The X^2 test indicated that the decrease in percentage survival of the fetal-inoculated mice over each of the control groups was statistically significant ($P<0.001$). Table 4 gives the incidence and X^2 analysis of tumors in adequately studied individual sites of animals that survived 2 months or longer. In all sites except the subcutaneous tissue, the incidence of tumors was higher in the fetal-inoculated mice than in either the uncontaminated controls or contaminated controls. An increase in the incidence of tumors in fetal-inoculated mice was statistically significant by the X^2 test in 2 sites, when compared with uncontaminated controls (table 4, column 5), and in 6 sites when compared with contaminated controls (table 4, column 6). Some quantitative differences were noted in the extent of the tumors in the 3 groups, but in most tumor types, the number of lesions was too small and the ages of the mice varied too much to evaluate differences properly. The fetal-inoculated mice died earlier, not only because they had a greater variety of tumors but also because

[9] Mr. Nathan Mantel and Miss Joan Gurian assisted with the statistical analysis.

TABLE 4.—Incidence of tumors in adequately studied organs of animals that survived 2 months or longer*

	Sites with tumors/total sites examined			X^2 test of significant difference between:			
	I. Fetal-inoculated group (*contaminated mothers*)	II. Newborn-inoculated group (*uncontaminated mothers*)	III. Newborn-inoculated group (*contaminated mothers*)	Groups I, II, III	Groups I and II	Groups I and III	Groups II and III
A: Salivary glands	17/25	11/17	1/17	***		***	***
B: Renal cortex	21/28	8/17	1/18	***		***	*
C: Mesothelium	19/28	7/17	6/18	*		*	
D: Thymus	3/27	0/17	0/18		+	+	−
E: Breast	9/27	2/17	1/18	*			
F: Subcutaneous tissue	0/28	0/17	2/18		−	+	+
G: Bone	20/25	4/17	6/17	***	***	**	
H: Renal pyramids	17/27	2/17	4/18	***	***	*	
I: Hair follicles	5/25	0/17	0/18	*	+	+	−
J: Thyroid	6/18	3/14	0/16	*		*	+
K: Adrenal	3/19	0/16	0/17		+	+	−

* An analysis of the total lesions per animal, taking into account the sites examined, also clearly indicated ($P<0.001$) the 3 groups to be different in the over-all frequency of occurrence of lesions. Chi-square tests were computed on the total number of mice. In principle it would have been more appropriate to consider individual litters. However, the data in tables 2 and 3 indicate no important litter differences within a group. The average number of lesions per mouse is uniformly high, moderate, and low for the litters of the respective groups I, II, and III.

* = Difference significant at the 5 percent level.
** = " " " 1 percent " .
*** = " " " 0.1 percent " .
\+ = Difference as great as can be obtained from the data.
− = No tumors in either group tested.

they had tumors that affected more vital organs. Tumors of the bone and renal pyramids were much more common in the fetal-inoculated group than in the control groups and seemed to account for most of the early deaths.

Differences were also evident between the 2 groups of mice inoculated as newborns (tables 3 and 4). The increase in percentage survival, at 7 months, of the contaminated-control group over the uncontaminated-control group (67% *vs.* 35%) was large, though the small number of mice did not indicate a statistically significant difference. Even though a greater number of animals in the contaminated-control group survived longer than 7 months, this group developed fewer tumors. The difference was highly significant for salivary-gland tumors and was significant at the 5-percent level for renal tubular lesions (table 4, column 7). Complete regression of the renal tubular lesion in older animals might account for the lower incidence of this lesion in the contaminated controls, but salivary-gland tumors could hardly regress without leaving more residual damage than was observed. All the mice in the uncontaminated-control group that developed tumors had salivary-gland tumors. This is in accordance with past experience with Swiss mice from the Jay-Poiley colony that had been inoculated, as newborns, with similar preparations (*3*). But only 1 animal in the contaminated-control group had a salivary-gland tumor. It should be noted also in relation to our past experience that the fetal-inoculated group, which was also from contaminated mothers, had 5 mice that survived to maturity, responded by developing some of the variety of tumors, but failed to develop salivary-gland tumors (table 2).

DISCUSSION

The analysis of the incidence of the various lesions and of the morphologic impressions regarding the extent of the lesions indicates that for some tumors the quantitative response was greater in the mice inoculated as fetuses than in mice inoculated as newborns. The state of development at the time of inoculation is apparently significant, though this experiment does not show that such factors are responsible for the selective sites of tumor formation.

The data are, perhaps, more significant in regard to the importance of the virus in the formation of the tumors. The mice inoculated as newborns from mothers exposed to an environment contaminated by polyoma virus had a significantly longer lifespan and lower incidence of salivary-gland tumors and of renal tubular lesions than either the mice inoculated as newborns from uncontaminated mothers or mice inoculated as fetuses from contaminated mothers. Furthermore, some tumor-bearing animals in the latter group also failed to develop salivary-gland tumors. Some factor capable of preventing the development of these proliferative lesions must have been available to the newly born mice of mothers bred in a contaminated environment, though this factor did not seem to be as effec-

tive if the animals were inoculated *in utero*. Stewart *et al.* (*13*) have shown that salivary-gland tumors do not develop within a 4-month period if, before inoculation, the polyoma tissue-culture inoculum is mixed with serum from mice that have been exposed to an environment of polyoma-infected animals. However, if the transfer of maternal antibodies through the placenta inhibited the effect on the newborn mice in our experiment, it is difficult to understand why this should not have been as effective in the fetuses. Developmental immaturity might have acted to obscure a decrease in the incidence of tumors in the fetal-inoculated animals, or viral-inhibiting antibodies from the mother's milk might have enhanced the inhibition of salivary-gland tumor formation in animals inoculated as newborns. In either case, if the inhibition of tumor formation is related to the passive transfer of maternal antibodies to the newborn animal, it seems evident that the mothers could possess antibodies only to a contagious agent. In this regard it is interesting to note that in animals inoculated as newborns, which failed to develop salivary-gland tumors, apparent inhibition, though not complete, resulted also in a lower incidence of other tumors or in tumors that were either delayed in development or showed less pronounced degrees of cellular activity. This supports the thesis that tumors develop only if the virus becomes established in the host.

Other observations indicate that a virus may be closely associated with cells that form the tumors. The epithelial cells of organs, in which tumors develop initially, show degenerative changes and also intranuclear inclusions of the type commonly associated with viral diseases. And so far, histologic studies on animals treated with potent material after ultracentrifugation, animal passage and limiting dilutions (*13*), and plaque isolation (*24*, *25*) have failed to separate the agent responsible for the tumors from the agent responsible for these early cytologic changes. However, lesions of this type do not necessarily indicate that the tumors develop directly from the cells parasitized by the virus. The cells showing such changes appear incapable of dividing and the cells of the fully developed tumors do not show such changes. Even though it has been shown that the virus can be recovered from some of the tumors (*3*), the cells parasitized by the virus could yield a product of altered cell metabolism that is capable of stimulating cell proliferation. It is important to emphasize, even on the basis of this scant evidence, that mechanisms other than cell parasitization may be involved in the initiation of the tumors.

Aside from a consideration of the factors responsible for the tumors, the nature of these neoplasms requires critical study. The tumors in the various organs are peculiar not only because they are unlike those commonly observed in the mouse but also because the tumors in different organs show interrelated patterns of development, structure, and behavior. We have referred previously to the similar nuclear alterations that have been found in the epithelium of the salivary glands, breast, hair follicles, thyroid glands, thymus, and adrenal glands of treated mice. The tumors that form in these organs all contain proliferative epithelial components,

but the masses of the tumors are composed largely of rapidly proliferating undifferentiated cells that do not have a clearly identifiable origin and may represent immature cells that have more than one pathway for differentiation. This same complex of epithelial-cell proliferation *in situ* and subsequent outgrowth of undifferentiated cells occur also in the kidney, though in the kidney the epithelial changes in the tubules of the cortex are far removed from the massive mesenchymal cell proliferation that occurs in the papilla. The tumors in organs covered by mesothelial cells or osteogenic cells represent a type of response that is equally extraordinary. So far we have not seen nuclear alterations in the cells of these organs, but the rapid growth of undifferentiated cells is much the same, and in these areas, differentiation into fibrous tissue, fat, or bone occurs.

The behavior of the tumors depends primarily on the activity and potentialities of these undifferentiated cells. The tumors grow centrifugally as nodular cell masses that follow the structural confines of the organ in which they arise. Where boundaries do not exist, growth appears to be in the direction of least resistance, which accounts for the polypoid structure of the tumors of the kidney, pleura, and even bone. In some tumors, particularly those of the breast and salivary glands, detached cells are carried to pulmonary vessels where they continue to grow to a limited extent. In this experiment, only the bone tumors, and few of these, seemed to possess the ability to destroy tissues by direct invasion, and definite lung metastases resulted from only one bone tumor and one renal tumor. It is also clear that cell growth in the tumors of some sites can become less active and that the tumors can regress. The densely sclerosed pleural tags and the irregular osteoid deposits on bony surfaces that are found in older mice are strong indications of involution. In the tumors of the salivary glands and renal pyramids, regression is suggested by the extensive sclerosis which sometimes occurs, even in the smaller tumor nodules. In one mouse the metastasis from a renal tumor showed sufficient scarring to suggest regression.

The single quality of neoplasia that predominates in the tumors, whether they are large or small, or are seen in young or old mice, is the rapid proliferation of circumscribed masses of undifferentiated cells. The characteristics that are generally attributed to malignant or autonomous neoplasms, such as aberrations in cell structure and mitotic apparatus, and the invasive destruction of tissues in local and distant sites, are lacking in nearly all the tumors. In their activity, the tumors are somewhat analogous to the papillomatous tumors of the skin of rabbits that occur after either chemical or viral application (*26*), but because of the distinctive immaturity of the cells in the tumors that follow polyoma inoculation, it is even more difficult to evaluate evidence of progression to malignancy than it is in skin tumors of rabbits.

In the opinion of the senior author, it is probable that most of the tumors represent growth that is dependent on a continuing stimulus and in this sense represent hyperplasia or conditioned neoplasia, as defined by some morphologists (*27*, *28*). Further experiments are necessary to determine

the etiologic association between the initial and perhaps continuing stimulus, which seems almost certainly related to viral parasitization of host cells and the progressive acquisition of those attributes of tumors that indicate an altered reactivity of a more aggressive nature.

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PLATES

526279—59——25

Other illustrations of tumors induced by similar means can be found in (*1*), (*5*), (*6*), and (*21*). All sections are stained with hematoxylin and eosin unless otherwise stated. Photomicrographs by Mr. Gebhard Gsell.

PLATE 143

FIGURE 1.—Area of small tumor nodule in submaxillary gland of 5-month-old mouse inoculated after birth. Acini have been obliterated by proliferating undifferentiated cells. Glandular structures are hyperplastic intercalated ducts. × 380

FIGURE 2.—Medium-sized tumor nodule in the parotid gland of a 3-month-old mouse inoculated before birth. Proliferating cells have assumed fusiform shapes and crowded the ducts into periphery of lobules within nodule. × 200

FIGURE 3.—Area from circumscribed parotid-gland tumor nodule measuring 30 mm. in diameter in 5-month-old mouse inoculated before birth. Polypoid cell masses can be seen protruding into vascular lacunae. Intravascular deposits of this tumor were present in lungs. × 108

FIGURE 4.—Parotid-gland tumor nodule with marked sclerosis in 3-month-old mouse inoculated before birth. Hyperplastic ducts remain, but only a few tumor cells are in collagenous matrix. × 18

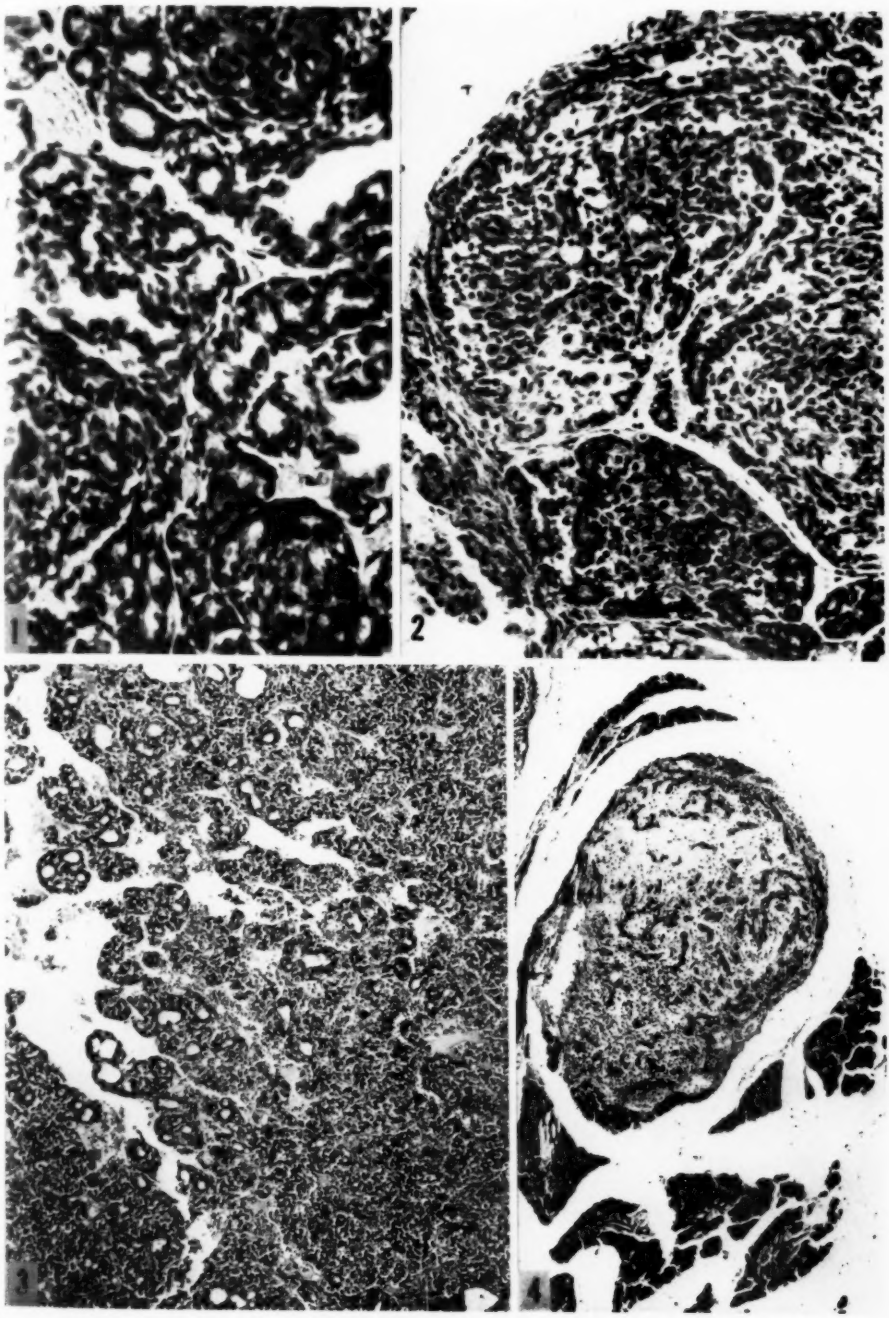


PLATE 144

FIGURE 5.—Proliferative renal tubule lesion in 4-month-old mouse inoculated after birth. Marginal leukocytic infiltrations were common in older mice. × 340

FIGURE 6.—Detail of renal tubule cells in 3-month-old mouse inoculated after birth. Distal tubule at *top* is proliferating lesion; hypertrophied cell at *bottom* has an acidophilic intranuclear inclusion. Masson's stain. × 1,700

FIGURE 7.—Two stages in development of hypertrophied renal tubule cells in 4-month-old mouse inoculated after birth. Cell at *top left* has an acidophilic intranuclear inclusion. Cell at *bottom left* has central homogeneous intranuclear mass with marginated chromatin. Cells on *right* side are in unaffected proximal convoluted tubule. Phloxin-tartrazine stain. × 1,700

FIGURE 8.—Renal tubule cells with intranuclear inclusions and degenerating cells with extranuclear acidophilic bodies similar to inclusions in 2-month-old mouse inoculated before birth. Phloxin-tartrazine stain. × 1,700

FIGURE 9.—Renal tubule cell with marginated chromatin and halo around acidophilic intranuclear inclusion body in 17-day-old mouse inoculated before birth. × 1,700

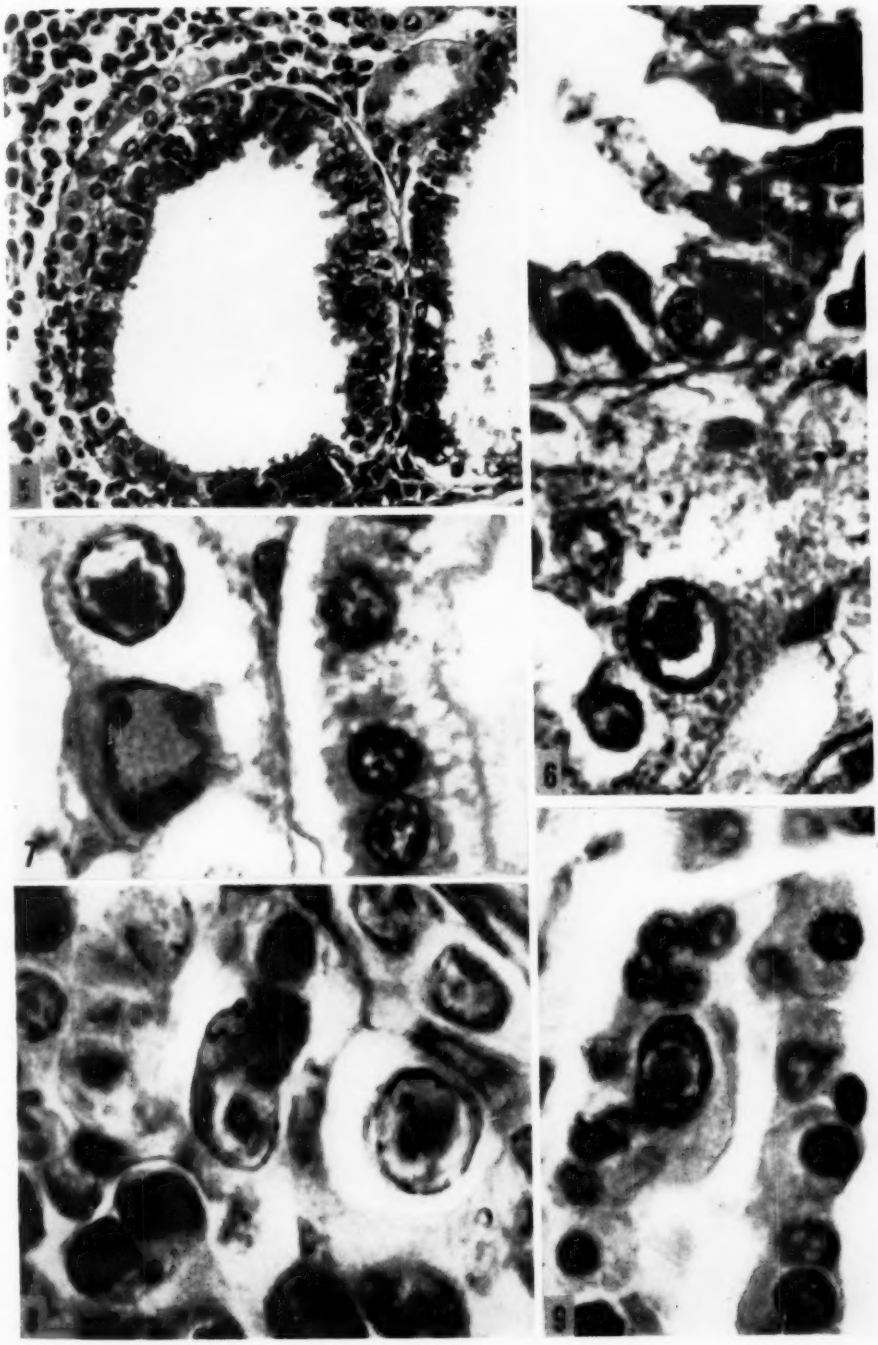


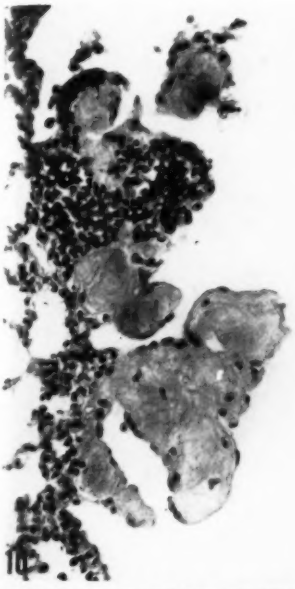
PLATE 145

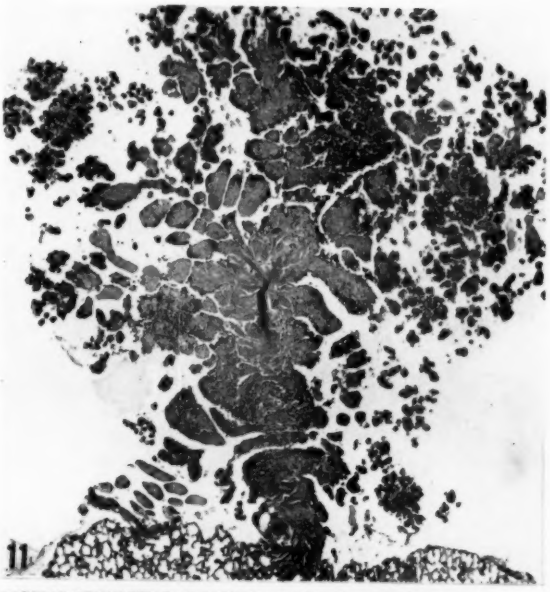
FIGURE 10.—Complete sclerosis of pleural tumor in 6-month-old mouse inoculated after birth. Infiltrations of mononuclear leukocytes and hemosiderin were often seen in atrophic lesions. × 210

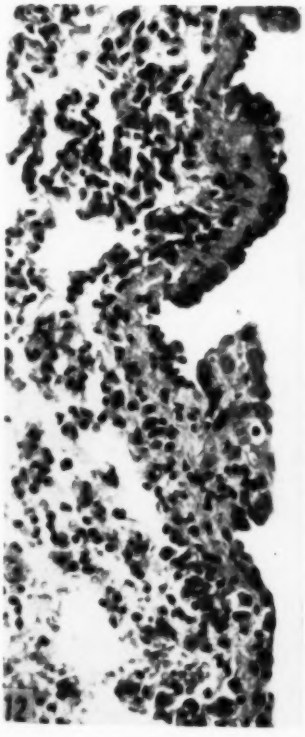
FIGURE 11.—Massive polypoid pleural tumor in 4-month-old mouse inoculated before birth. × 30

FIGURE 12.—"Early" pleural tumor in 2-month-old mouse inoculated before birth. Focal hyperplasia of mesothelium merges with small polyp. × 290

FIGURE 13.—Thymic tumor in 4-month-old mouse inoculated before birth. Organoid glandular development merges with less discrete reticular cell proliferation. Normal thymic cortex is illustrated in *lower right*. × 125







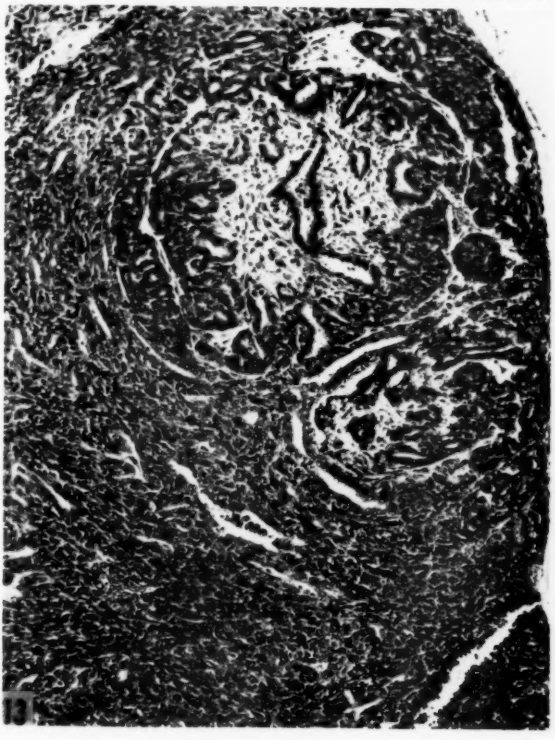


PLATE 146

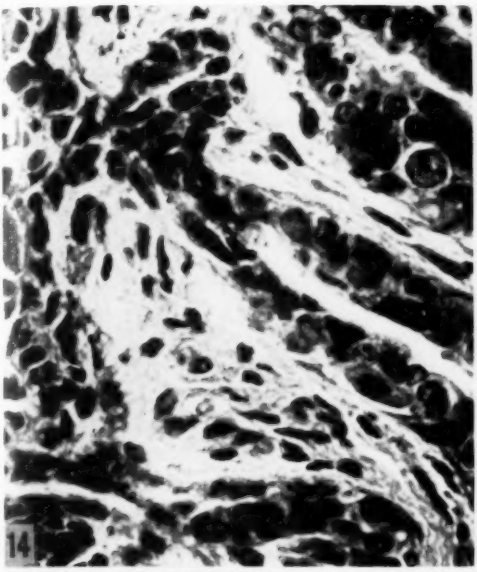
FIGURE 14.—Mammary ducts, in 13-day-old mouse inoculated before birth, showing hypertrophied lining cells with homogeneous intranuclear masses and marginated nuclear chromatin. × 690

FIGURE 15.—Mammary tumor, in 3-month-old female mouse inoculated before birth, showing papillomatous masses lined by duct epithelium and composed of uniformly undifferentiated tumor cells. × 108

FIGURE 16.—Osteogenic tumor of vertebra invading spinal cord in 4-month-old mouse inoculated before birth. Marked involvement of the marrow cavity and osteosclerosis of vertebral body and lamina can be seen. Entire lesion was limited to single vertebra. × 16

FIGURE 17.—"Early" osteogenic tumor in nasal-turbinate bone in 4-month-old mouse inoculated after birth. Bone tumor lies above tumor of submucosal gland; such collision tumors were not uncommon findings. × 100

FIGURE 18.—Cell structure of osteogenic tumor of maxilla in 4-month-old mouse inoculated before birth. Tumor has less osteoid and more evidence of cell proliferation than tumors in figures 17 or 19. × 315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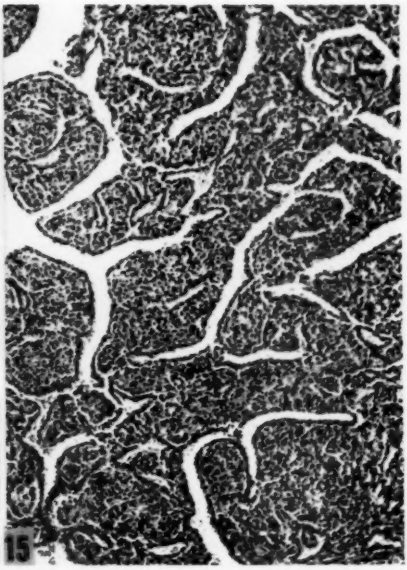
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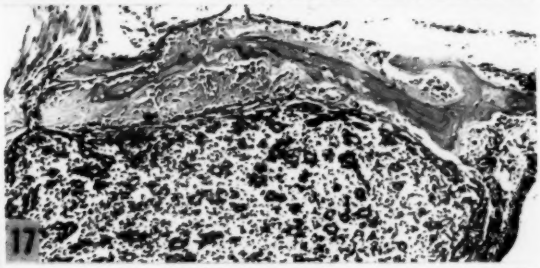
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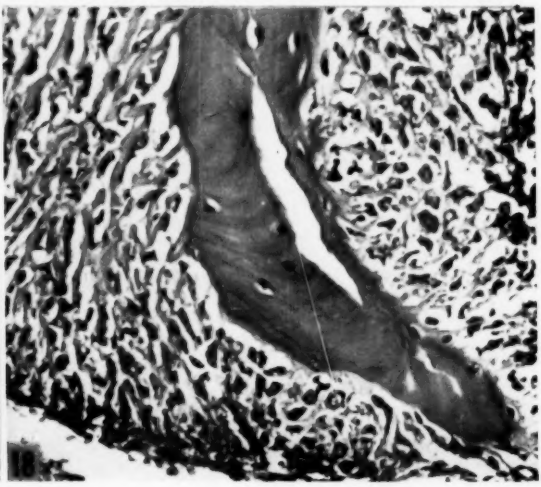
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PLATE 147

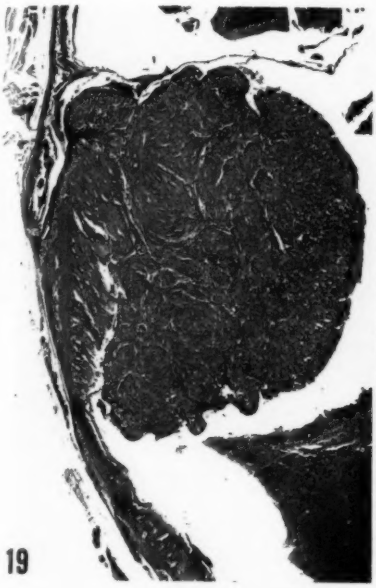
FIGURE 19.—Massive osteophytic tumor of inner table of calvarium of 4-month-old mouse inoculated before birth. Except for periphery, tumor is largely composed of osteoid containing only isolated osteoblasts. × 14

FIGURE 20.—Detail of osteophytic tumor 10 mm. in diameter from shoulder girdle of 4-month-old mouse inoculated before birth. Tumor was largely composed of lipoblasts in which islands of osteogenic tissue were formed. × 150

FIGURE 21.—Tumor of renal mesenchyme in 3-month-old mouse inoculated before birth. Tumor formed polypoid masses which grew into pelvis of kidney. Masson's stain. × 12

FIGURE 22.—Detail of cell type in renal mesenchymal tumor illustrated in figure 21. *Note* hyperplastic vascular components and moderate sclerosis. × 150

FIGURE 23.—Two mesenchymal tumors in renal medulla of 6-month-old mouse inoculated before birth. One tumor was composed almost entirely of scar tissue. Other tumor contained areas of degenerating spindle cells but also had marginal areas of sclerosis. × 12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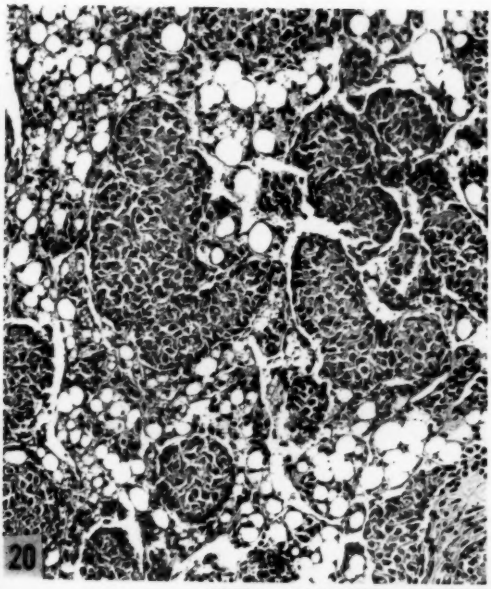
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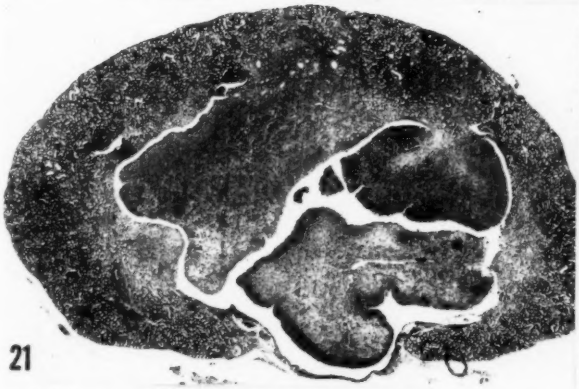
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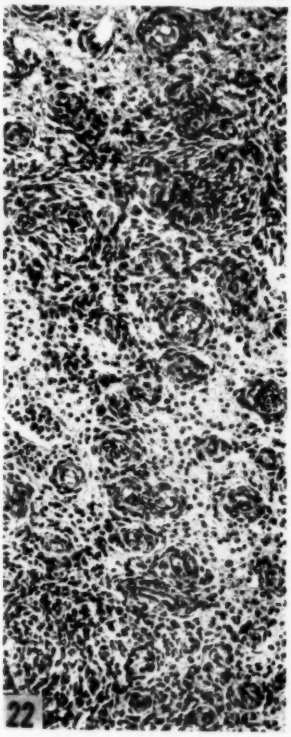
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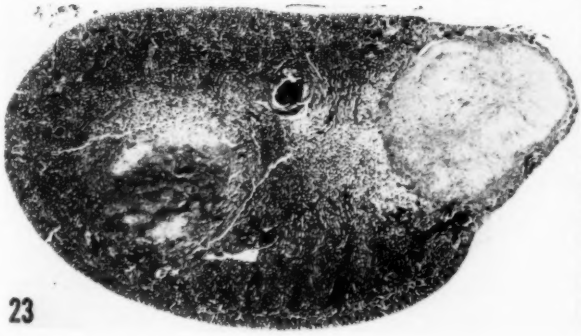
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PLATE 148

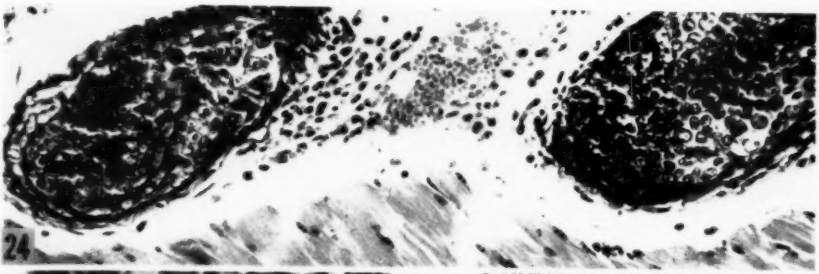
FIGURE 24.—Small hair-follicle lesions in 4-month-old mouse inoculated before birth. Lesions were confined to dermal hair sheath and were composed of hypertrophied cells with nuclear alterations. × 210

FIGURE 25.—Intranuclear inclusions in cells at base of hair follicle in 3-month-old mouse inoculated before birth. × 880

FIGURE 26.—Papillary epithelial tumor of thyroid gland in 4-month-old mouse inoculated before birth. A few areas of cell proliferation can be seen between glands. A thin fibrous capsule completely surrounded tumor. × 95

FIGURE 27.—Hypertrophied cells of thyroid follicles from margin of papillary tumor illustrated in figure 26. Hypertrophied marginal cells at *bottom* contain intranuclear inclusions, proliferating tumor cells at *top* do not. × 880

FIGURE 28.—Circumscribed epithelial tumors of thyroid gland in 3-month-old mouse inoculated before birth. Proliferating cells can be seen around follicles both inside and outside adenomatous nodules. × 165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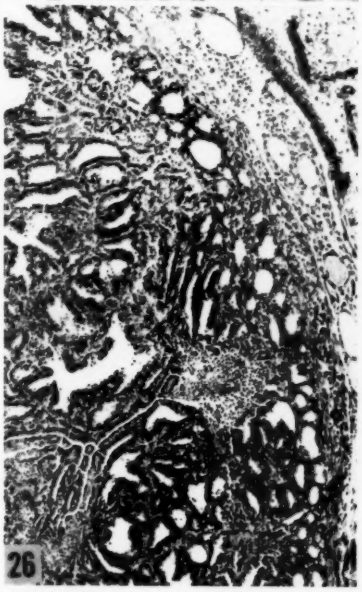
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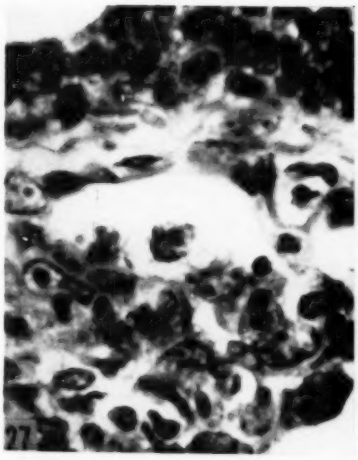
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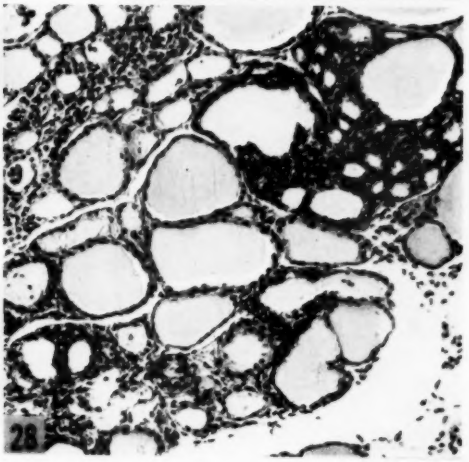
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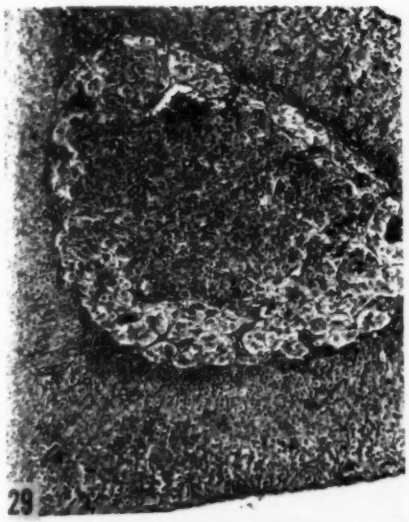
PLATE 149

FIGURE 29.—"Early" tumor of adrenal gland in 3-month-old mouse inoculated before birth. Tumor was confined to medullary zone. × 85

FIGURE 30.—Massive tumor of adrenal gland in 3-month-old mouse inoculated before birth. Tumor was confined by capsule of adrenal gland. Mesenchymal tumor of renal pelvis is shown on *right*. Neither tumor had involved intervening lymph node. × 12

FIGURE 31.—Detail of tumor illustrated in figure 29. Many cell nuclei are filled with thick chromatin bars and mitotic spireme formations. × 1,700

FIGURE 32.—Detail of tumor illustrated in figure 30. Two cell types are represented. Tumor was primarily composed of large epithelial cells which were often in mitoses. Cells that make up fibrovascular trabeculae are smaller and more uniform in configuration. × 430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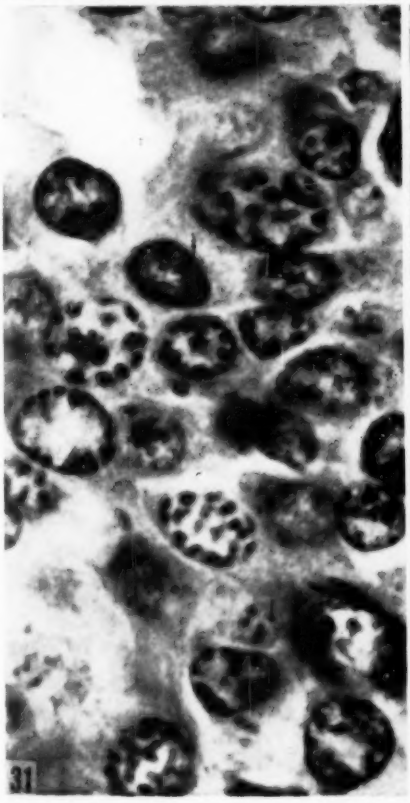
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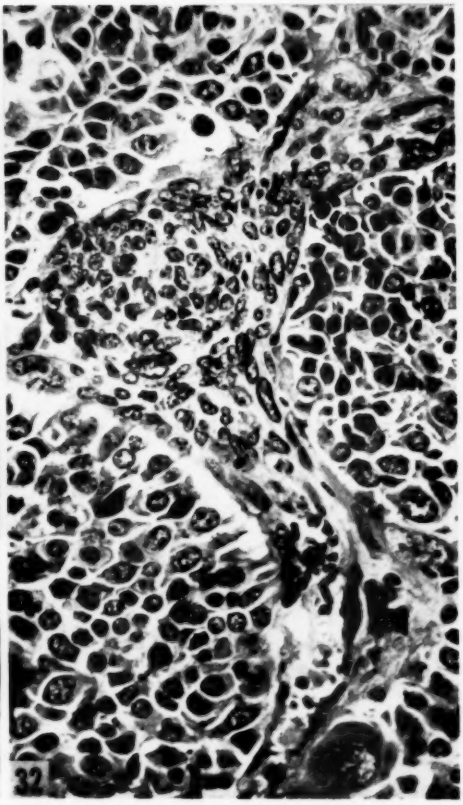
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Extent of Nonspecific Background Localization of Radioiodinated Serum Proteins in Human Tumor and Normal Tissues [1]

EUGENE D. DAY *and* AHSEN ÖZARDA,[2] *Departments of Biochemistry Research and Nuclear Medicine, Roswell Park Memorial Institute, Buffalo, New York*

SUMMARY

The 24-hour uptake of intravenously injected, radioiodinated, rabbit- and human-serum globulins and human-serum albumin, in a variety of human tumor and normal tissues, was measured after surgical excision. No detectable difference between the uptake by tumor and normal tissue was found. An average background localization value of 0.004 percent (normalized to a 50-kg. patient) of the injected radioactive dose per gm. of wet, unperfused tissue was obtained. It was concluded that in any future assays of whole radioiodinated antitumor antiserum globulin, tumor-localizing antibodies of average titer, if and when obtained, should be detectable in human tumors, above and beyond the nonspecific background localization of radioiodinated serum proteins, without purification and concentration.—J. Nat. Cancer Inst. 23: 1477–1482, 1959.

IN ORDER to demonstrate the specific localization *in vivo* of radiolabeled antibody in human tumors, one should have knowledge of the background localization of radiolabeled control proteins. This can be best obtained with the paired radiolabel technique in which, in a given subject, antibody and control are assayed together (*1*). To choose suitable conditions for the preparation and assay of such a pair, however, since there may be only one chance in a given human subject for such an assay, one should also be able to predict with a fair amount of reliability, prior to use of the pair, the degree to which an antibody globulin would have to localize in human tumors to be recognized above and beyond the background of the normal control protein. Would purification and concentration of an antibody be necessary as in localization in transplanted rat tumors (*2*), or could a whole, unpurified antiserum globulin, of which antibody might comprise as little as 1 percent, be assayed for the presence of antibody as in the localization in rat kidney (*3*)? How much variation would occur from tumor to tumor and from patient to patient? What would be the minimum amount of radioactivity to be injected, which would give a reliably countable amount of radioactivity in samples obtained for assay

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[2] The authors wish to thank the members of the Departments of Surgery and Pathology for their cooperation in this survey.

after surgical excision? How uniform would the distribution of control protein be throughout a tumor?

From the results of Stirrett, Yuhl, and Libby (*4*, *5*), one might have suspected that liver metastases, at least, would localize radioiodinated, nonspecific human-serum proteins preferentially. Such a high, nonspecific localization would pose a problem as far as assay of antibody over control protein is concerned. However, the negative results of Madell *et al.* (*6*) who tried to repeat this work, and the recent report of MacEwan (*7*), who showed that no selective concentration of serum albumin occurred in the liver metastases of 4 terminal cancer patients, lead one to suspect that perhaps some impurity was present in the serum albumin used by Stirrett *et al.*, which accounted for the localization they observed. In any event, even in their work, primary tumor lesions in liver showed no localization of labeled albumin over that of normal tissues. Since each of these 3 reports was concerned only with the ratio of radioactivity between tumor and normal tissue, no data were given with regard to actual concentration of radiolabeled protein and distributions of injected radioactive doses appearing in the tissues.

In the present short survey, the 24-hour uptake of intravenously injected rabbit- and human-serum globulin and human-serum albumin, in a variety of human tumor and normal tissues, was measured after surgical excision. The results here indicate that there is no detectable difference between tumor and normal tissue with regard to this uptake. The nonspecific background localization of normal serum albumin and globulin in human tumors appears to be like that of corresponding normal tissues, a result unlike that obtained with transplanted rat tumors (*2*). In addition, the results reveal a surprisingly high degree of uniformity in background localization from tumor to tumor and patient to patient—as uniform, at least, as from sample to sample within a given tumor or normal organ. Therefore in any future assays of antibodies localized *in vivo* in human tumors, decisions posed by the foregoing questions will be much easier to make.

MATERIALS AND METHODS

Rabbit γ-globulin was from a lyophilized pool separated from normal rabbit serum by a sodium sulfate fractionation procedure described previously (*8*). For injection in humans, each solution was passed, after radioiodination, through a sterile, ultrafine, sintered-glass filter. Patients selected to receive this material had reacted negatively when skin-tested for allergic response to the globulin.

Human-serum globulin for patients T.D. and C.S. was from a lyophilized pool separated from normal human serum by sodium sulfate, but the fraction contained α- and β- as well as γ-globulins by precipitation from 18 percent (w/v) sodium sulfate. After radioiodination each preparation was passed through a sterile, ultrafine, sintered-glass filter.

The human-serum globulins for patients A. F., H. S., G. S., and A. C. were prepared by this same procedure from the individual serums obtained from each patient a few days before their operation. The globulins were radioiodinated by methods previously described (*8*, *9*), so that a final, sterile, 4-ml. dose in saline contained 30 μc. and 1 mg. of the radiolabeled protein.

Radioiodinated human-serum albumin, obtained from Abbott Laboratories, was diluted with sterile saline for injection. Each 4-ml. dose contained approximately 40 μc. and 1 mg. of the radiolabeled protein.

Each patient received an intravenous injection 17 to 26 hours before undergoing surgery. The tumor samples for counting represented about one third the total tumor mass in each case; normal tissues, about one third the total amount removed, at the time of surgery, from near the tumor site.

The tissues were rinsed in saline, blotted, weighed, and then cut into 1-gm. portions. They were assayed for radioactivity by counting in a well-type scintillation counter with a sodium iodide crystal and a 3-inch lead shield. The counter had a counting efficiency for I^{131} of 40 percent and a normal background of 120 cpm. The average sample, 0.003 percent of the injected dose, counted approximately 1000 cpm; a minimum of 2000 counts per sample was obtained and a minimum counting time of 1 minute per sample was maintained. Whole blood obtained, at most, a few hours after operation was counted in 1-ml. portions.

RESULTS AND DISCUSSION

The data are summarized in table 1. Regardless of size, location, or type of tumor or of the type of radiolabeled serum protein for assay, the uptake per gm. after 1 day was about 0.004 percent of the injected dose per gm. in a 50-kg. patient, or 0.003 percent in a 75-kg. patient. The average uptake per gm. of normal tissue was not statistically different. The ratio between the doses in normal and tumor tissue for each patient averaged 0.92 ± 0.36 percent for 14 of the patients. Considering the fact that tissues were not perfused, were weighed as wet samples, and extended over a variety of vascular types, and that the patients ranged in age from 47 to 83 years and in weight from 46 to 72 kg., such values appear to be surprisingly uniform. Moreover, there is as much uniformity among the 15 tumors as there is within a single one. Table 2 gives the distribution of radioactivity among the 1-gm. portions of a 60-gm. stomach tumor and corresponding normal stomach. The range of values within these tissues extended well over the range of values for all tissues given in table 1.

The background localization value of 0.004 percent per gm. in a 50-kg. human would be comparable, in a 5-kg. rabbit, to 0.04 percent per gm.; in a 200-gm. rat, to 1.0 percent per gm.; and in a 25-gm. mouse, to 8.0

TABLE 1.—Nonspecific localization of radioiodinated serum proteins in human tumor and normal tissues after 1 day

Iodinated protein injected	Patient			Tumor tissue		Normal tissue		Localization per gm. of tissue* (*percentage of injected radioactive dose*)		
	Initials	Age and sex	Weight (*kg.*)	Type (*final diagnosis*)	Weight† (*gm.*)	Type	Weight† (*gm.*)	Tumor	Normal	Blood
Rabbit-serum globulin	S. E.	62 M	50. 7	Squamous-cell carcinoma, nose	0. 24	Nasal mucosa and skin	0. 64	0. 0035	0. 0036	0. 011
	H. P.	55 F	56. 3	Carcinoma, mammary gland, ulcerated	0. 59	Muscle and breast	1. 43	0. 0037	0. 0025	0. 019
	B. M.	55 F	55. 8	Carcinoma, mammary gland	0. 84	Breast	0. 39	0. 0040	0. 0028	0. 021
	T. D.	73 M	72. 1	Adenocystic carcinoma, tongue	0. 50	Tongue	0. 50	0. 0032	0. 0026	0. 013
Human-serum globulin	A. F.	65 F	69. 1	Carcinoma, stomach	59. 8	Stomach	14. 0	0. 0053	0. 0053	0. 016
	C. S.	49 F	52. 2	Fibrocystic disease, mammary gland, benign	0. 82	—	—	0. 0073	—	0. 022
	H. S.	60 M	52. 8	Carcinoma, rectum	0. 61	Rectal mucosa and liver	1. 58	0. 0027	0. 0038	—
	G. S.	46 F	67. 1	Carcinoma, rectum	13. 0	Colon	14. 3	0. 0043	0. 0038	0. 015
	A. C.	63 M	57. 6	Recurrent carcinoma, colon	106. 0	Colon	59. 0	0. 0032	0. 0032	0. 011
	M. H.	50 F	55. 2	Pseudomyxoma, peritoneum	22. 4	Uterus	8. 60	0. 0023	0. 0020	0. 013
Human-serum albumin	J. F.	80 F	62. 7	Carcinoma, mammary gland	13. 0	Breast, adipose	8. 99	0. 0043	0. 0015	0. 016
	B. L.	51 F	46. 0	Carcinoma, ascending colon	5. 62	Colon	2. 30	0. 0047	0. 0079	0. 015
	T. H.	47 F	55. 4	Leiomyosarcoma, liver, metastatic	11. 5	Liver	13. 0	0. 0035	0. 0030	0. 007
	W. S.	55 M	60. 0	Squamous-cell carcinoma, larynx	0. 54	Larynx	1. 35	0. 0041	0. 0050	0. 006
	H. G.	83 M	66. 8	Mesenchymoma, thigh	2. 76	Thigh muscle	3. 81	0. 0033	0. 0012	0. 015
							Mean	0. 0040	0. 0034	0. 014
							Standard deviation	±0. 0012	±0. 0017	±0. 005

*Normalized to 50-kg. subject.
†About one third of total surgical specimen.

TABLE 2.—Distribution of radioactive human-serum globulin in carcinomatous and normal stomach, patient A. F.

Range (*percentage of injected radioactive dose*)	Number of 1-gm. pieces containing dose within range	
	Tumor	Normal
0.0001–0.0010	0	0
0.0011–0.0020	3	0
0.0021–0.0030	6	1
0.0031–0.0040	8	2
0.0041–0.0050	9	3
0.0051–0.0060	15	4
0.0061–0.0070	9	2
0.0071–0.0080	6	2
0.0081–0.0090	3	0
0.0091–0.0100	1	0

percent per gm. Such values are about twice those actually obtained in these animals in *unperfused* tissues, after 24 hours. If we were studying laboratory animals rather than patients, we would undoubtedly have carried out the assays 3 to 7 days after injection to permit the background localization to drop to much lower values. In patients of the type studied, however, for whom operation schedules can not be made with much assurance several days in advance, it is practical to obtain the 24-hour data as we have done here.

Even with the rather high background encountered here, a tumor-localizing antibody-globulin preparation would easily be recognized if it assayed over 0.01 percent per gm. For example, a whole globulin that might contain 1 percent localizing antibody (some whole antikidney antiserum globulins assay as high as 3 percent), if distributed evenly, would be recognized as containing antibody even in as large a tumor as 100 gm. It can be expected, therefore, that except for antiserum globulins of extremely low titer and tumors of extremely large size, localizing antibodies in general should be detectable in human tumors when the whole radioiodinated globulin containing them is injected 24 hours before surgical removal of the tumor.

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*Normalized to 50-kg. subject.
†About one third of total surgical specimen.

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